Regeneration in the aging peripheral nervous system

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Abstract

In the peripheral nervous system (PNS), aging is associated with a number of disorders, including a decline in regenerative capacity after injury. Although this decline has been observed in both rodents and humans for decades, the cellular and molecular underpinnings of this defect have remained elusive. As such, the goal of this thesis was to elucidate, at least in part, how aging impinges on axonal regeneration.

In this work, we demonstrate a progressive, age-dependent slowing in the recovery of sensory and motor function following peripheral nerve injury. A systematic investigation of the possible causes follows, focusing on the potential roles of neuronal intrinsic growth, immune cell function, and Schwann cell responses. Unexpectedly, an array of *in vitro, in vivo* and *in silico* experiments provide no evidence that neuronal intrinsic growth is altered with aging. In addition, exposure of youthful circulating factors to aged mice fails to rescue the age-associated phenotype, implying that macrophage-intrinsic defects are unlikely to be involved. In contrast to these results, functional recovery in aged animals can be fully rejuvenated by transplantation of young nerve grafts.

Likewise, aged nerve grafts transplanted into young hosts results in diminished axonal regeneration. Microarray profiling of young and aged sciatic nerves reveals a highly perturbed transcriptional landscape, with the regulation of genes associated with the function of Schwann cells being particularly aberrant. These alterations are mirrored functionally, as aged Schwann cells fail to efficiently de-differentiate and engulf myelin. Finally, it is also noted that the protein expression of a key regulator of Schwann cell injury responses, c-Jun, is altered in aging nerves. Taken together, this thesis work shows that extrinsic factors are sufficient to rejuvenate age-associated decline in nerve regeneration, and suggest Schwann cells as key effectors of the aging PNS.

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CHAPTER ONE: INTRODUCTION

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PART I: SEARCHING FOR THE SCIENTIFIC FOUNTAIN OF YOUTH: WHY AND HOW WE AGE

"All children, except one, grow up."

- J.M. Barrie, Peter Pan

Introduction

As a literature and philosophy student in college, it is probably not a coincidence that I picked aging as a topic of study upon entering the field of biology. Is there any other subject, save for death and love, that has been so deeply and continually probed by artists and philosophers? From Buddha's recognition of aging as an inevitable part of human suffering, to Shakespeare's meditative sonnets on growing older, to Mark Twain's glib remark that, "Life would be infinitely happier if only we could be born at the age of eighty and gradually approach eighteen," the sense of loss that accompanies aging is a powerfully existential emotion, and one that has ignited countless efforts, from the spiritual to the scientific, to understand why we age and what we can do to thwart its ill effects.

Perhaps the most enduring anti-aging panacea was first described by the fifth century Greek historian Herodotus. Recounting a legendary race of people who lived on average to the age of 120, he described the secret to their longevity as,

"...a certain spring, in the water of which they washed became more sleek of skin..." (Herodotus, 1890). The myth of the Fountain of Youth, as it was later called, was so compelling that it was perpetuated for centuries, disseminating through the stories of Alexander the Great and Prester John before being carried whole heartedly into the middle ages by Ponce de Leon, a Spanish explorer who searched for the Fountain in what is now Florida (somewhat ironically the state with the highest percentage of senior citizens). Alas, while he was unsuccessful in his pursuit, there is perhaps some solace for the ghost of Ponce de Leon: the quest for the Fountain of Youth continues today, not as an archaeological treasure hunt, but as a scientific endeavor to understand the basic biological mechanisms of aging and develop therapeutics that extend health and lifespans.

From a healthcare standpoint, this endeavor has arguably never been more important than now. Diseases and disorders of aging represent a rapidly escalating worldwide problem. Currently, the worldwide average age is the highest in history, and the number of persons over the age of 65 is predicted to double over the next several decades (Caspari and Lee, 2004). This means large increases in age-associated diseases, including cancer, arthritis, osteoporosis, cardiovascular disease, and type-2 diabetes. Financially, this places a tremendous burden on the economy, where typical government systems of paying for the healthcare costs of the elderly, such as Medicare, become increasingly difficult as a greater percentage of the population is of advanced age. Indeed, neurodegenerative disorders represent major healthcare burdens,

where, for example, it is estimated that nearly 15% of persons over the age of 70 have dementia at a cost comparable to that of heart disease and cancer (Hurd et al., 2013). These statistics underscore the importance of both basic and translational age-related research.

Why do we age?

Despite tremendous advances in molecular biology over the last fifty years, aging remains in many ways a mysterious biological process. Although aging is a fundamental feature of most organisms, we still do not truly understand "why" we age in evolutionary terms. Part of this conundrum is the counterintuitive knowledge that aging is an evolutionarily conserved process, and yet, is thought to result from the absence of selective pressure. As such, there has been (and is still) considerable debate over a "theory" of aging that might explain all of its aspects while keeping in mind the guiding principles of Darwinian evolution. So far, many philosophical theories have been proposed, but none crowned the winner in the consensus of science.

Among the first theories of aging came from the German evolutionary biologist August Weisman, who proposed the "programmed death theory" in the late 19th century. According to Weisman, aging is essentially the process of eliminating inferior genetic material out of the population. In his view, each successive generation of a particular organism is a little "better" in an evolutionary sense,

being ever so slightly more adapted to its environment. Aging (and consequently death) thus serves to weed out the poorer genes of the population and clear the way for competitive resources such as food (Weisman, 1889). Though sensible on the whole, Weisman's argument fails to explain why aging happens gradually with time, as opposed to an immediate death. In addition, his theory does not account for the fact that, in the wild, most animals will never have the opportunity to die of old age; they will be killed by predators. Nevertheless, Weisman's attempt paved the way for others.

Over time, Weisman's programmed death theory expanded into the more modern "disposable soma theory," first articulated by Thomas Kirkwood in the 1970s (Kirkwood, 1977). Still frequently cited in literature today, Kirkwood argues that aging is the by-product of finite energy resources. With limited food, an organism cannot adequately allocate resources to both reproduction and the metabolically costly chore of maintaining and repairing somatic tissues. As a result, just enough energy is given to somatic maintenance for an organism to stay healthy through reproduction (and perhaps a bit longer in some cases to care for the young). Aging thus represents the accumulation of unrepaired somatic damage.

This is an intuitively attractive theory that fills in a major gap of Weisman's, namely in taking into account extrinsic mortality. Still, there are some notable flaws with the theory, such as the fact that reproduction is significantly more metabolically demanding for women than for men, and yet women have a

longevity advantage at all ages after birth (though this is not necessarily true for all species). In addition, one of the truly robust findings in the field of aging has been that restricting caloric intake extends lifespan. Clearly, this is the opposite of what disposable soma theory would predict when reducing the overall energy intake of an organism.

Contemporary theories

Today, understanding "why we age" is most often seen through the lens of two interrelated theories: mutation accumulation theory and antagonistic pleiotropy. Articulated by Medawar in 1952, mutation accumulation theory proposes that aging results from a decline in selection power against deleterious mutations that only manifest later in life (Medawar, 1952). Because animals are often killed in the wild, there is tremendous selection against traits that are deleterious at a young age due to loss of fitness. On the other hand, mutations expressed later in life are not selected against because they will often be passed down to the next generation before the effects of these mutations are observed. Thus, mutations will accumulate with aging that will not be selected against, leading to negative effects with advancing age. This conveniently explains why diseases such as Huntington's, which is the result of a mutation that manifests later in life, remain in the population. Experimentally consistent with these observations, flies that have been heavily inbred show dramatic increases in deleterious effects with age, largely as mutation accumulation theory might predict (Hughes et al., 2002).

Similarly, spontaneous mutations in flies tend to exert their negative effects within defined age-ranges (Pletcher et al., 1998).

The differences between mutation accumulation theory and antagonistic pleiotropy theory are slight but important. Whereas in mutation accumulation theory, deleterious traits develop because evolution essentially does not care about them, in antagonistic pleiotropy, these traits are actually directly selected for. This theory relies on the fact that many, if not all, genes are pleiotropic, likely exerting some beneficial effects and some negative ones. From an evolutionary point of view, a small beneficial effect early in life will thus greatly outweigh a large negative effect later in life. Again, this is because in the wild animals are likely to be killed, and thus there is significant selective pressure for genes that confer a benefit early in life. Perhaps one of the most illustrative examples of antagonistic pleiotropy is the tumor suppressor gene, p53. Mice with increased expression of p53 show better resistance to tumors, but their longevity is reduced (Jenkins et al., 2004; Tyner et al., 2002). Generally speaking, there is also a well-demonstrated inverse correlation between longevity and fitness. For example, although a mutation in DAF-2 doubles the lifespan of C. *elegans*, amazingly this allele is nearly eliminated from a population of worms in approximately four generations (Jenkins et al., 2004).

Overall, although it is interesting to philosophize about an evolutionary theory that will explain aging in grand unified terms, it is perhaps more plausible to

believe that no single idea will prove sufficient to explain all aspects of such a complex process. In this regard, it seems reasonable to think that mutation accumulation theory and antagonistic pleiotropy are not mutually exclusive, with both explaining slightly different potential mechanisms for why aging occurs.

How we age: fundamental pathways that regulate aging

For a long time, aging was considered to be a stochastic process, a kind of haphazard series of events that eventually leads to an equally random death. But, as is so often the case, "stochastic" tends to be a catch-all phrase in biology for processes we don't fully understand, and as we have elucidated pathways that regulate it, our view of aging as stochastic has faded.

Ironically, though this view is long passé, there is indeed some evidence to suggest that at least part of aging is indeed haphazard. In an intriguing study, Rea et al. demonstrated that individual C. *elegans* respond with different degrees of stress when exposed to an unwanted stimulus (Rea et al., 2005). Amazingly, the amount of stress induced in each worm, as indicated by a heat-shock protein reporter, predicted the longevity of these animals, with greater stress-responders living longer lives. Interestingly, the amount of stress-response in each animal was not heritable, demonstrating it was not the result of some genetic or epigenetic alteration, but rather a seemingly random response to an outside stimulus.

These data aside, it is somewhat surprising that there was such resistance to the idea that aging might be under genetic control given the extreme variability in the lifespans of different animals. For example, the mayfly lives 24 hours or less, while some species of tortoises can live for hundreds of years. Still, it took many years before conclusive evidence was provided that aging was subject to genetic regulation: in 1993, Cynthia Kenyon and colleagues published a paper demonstrating that a mutation in a single gene, DAF-2, which encodes for the insulin like growth factor (IGF-1) in worms, could double the lifespan of C. *elegans* (Kenyon et al., 1993). This was a landmark discovery that has since served as a cornerstone for all future age-related research.

Since demonstrating that a partial loss of function mutation in the insulin/IGF receptor increases the lifespan in worms, it has also been demonstrated in yeast, flies and mice (interestingly, complete absence of insulin signaling often results in reduced lifespan) (Kenyon, 2005). Furthering the significance of the insulin pathway in aging, other components of the insulin signaling pathway have been perturbed across a variety of species, all with the result that "turning down" insulin signaling has a beneficial effect on longevity **(Figure 1.1)**. For example, mutations that reduce AGE-1 (PI3K) also increase lifespan in worms (Morris et al., 1996) while over-expression of DAF-16/FOXO in drosophila extends lifespan (Giannakou et al., 2004). These findings extend, at least correlatively, to humans, where an over-representation of IGF1R mutations was found among a group of

female centenarians (Suh et al., 2008). Similarly, a deficiency in growth hormone receptor has been shown to correlate with reduced incidence of cancer and diabetes, but not necessarily lifespan (Guevara-Aguirre et al., 2011). Overall, it remains to be understood how exactly insulin signaling influences organismal aging, and whether it exerts its influence uniformly, through all tissues, or via certain master tissues or even cell types.

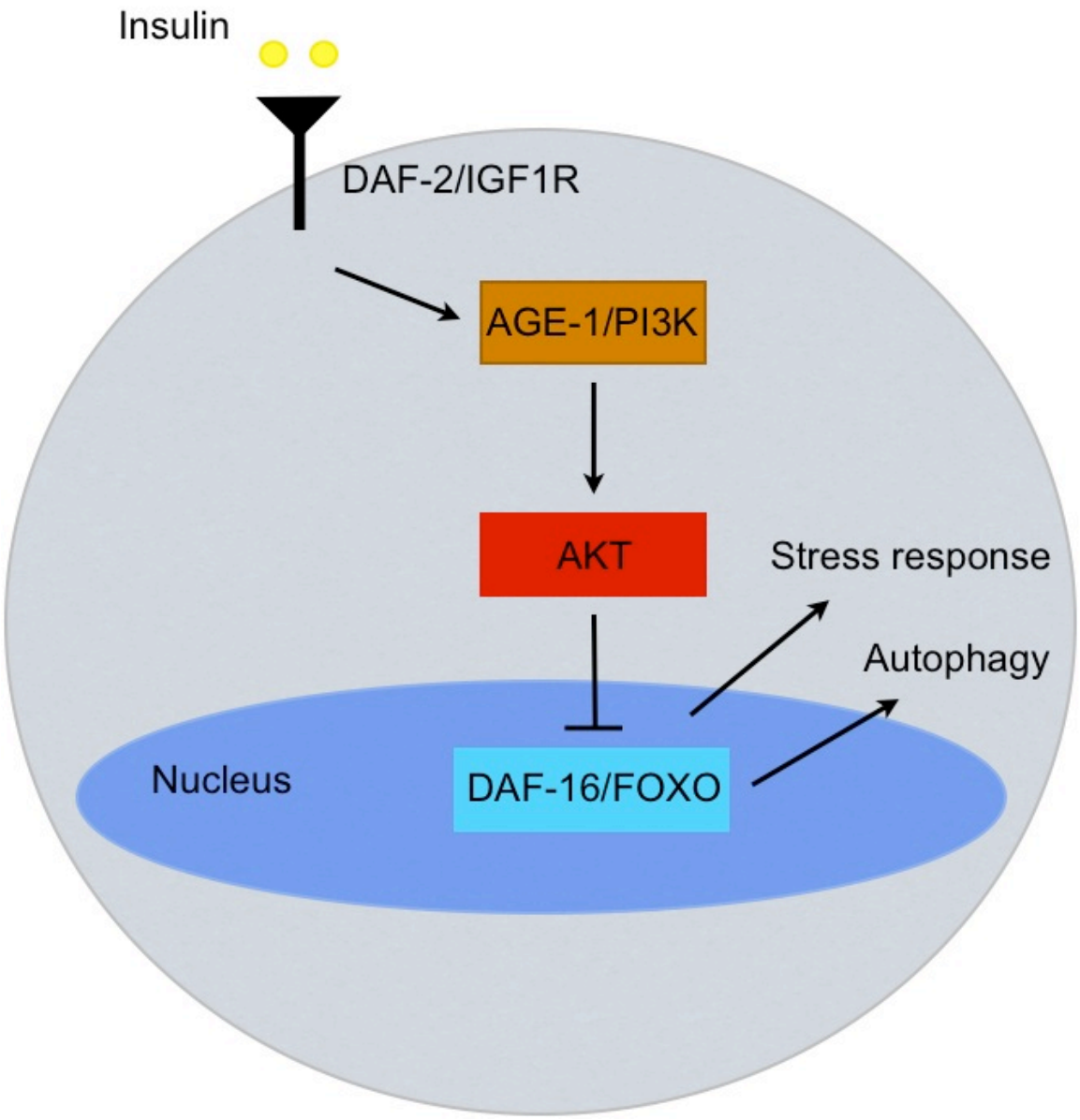

FIGURE 1.1: Main insulin signaling axis in aging. Insulin or growth hormone bind to the insulin receptor (DAF-2/IGF1R) leading to the inactivation of FOXO via AKT driven phosphorylation of DAF-2/FOXO. FOXO promotes a number of functions, including enhanced autophagy and increased stress response.

Other factors

One of the earliest discovered interventions that can modify longevity was caloric intake. In the 1930s, a seminal study demonstrated that reducing the amount of food rats consumed extended their lifespans (C.M. McCay, 1935). Since then, caloric restriction as a way of extending longevity has become one of the most reliable findings in the literature of aging, being reproduced in yeast, flies, worms, rodents and to some degree monkeys (still ongoing and still controversial) (Guarente, 2013). Interestingly, the mechanisms underpinning increased longevity from caloric restriction are not understood, but are variously thought to involve stress responses, insulin signaling or perhaps mediated via sirtuin activity (Guarente, 2013).

Cellular senescence is considered to be the state in which a cell has lost its proliferative potential while still remaining metabolically active. This can occur for a variety of reasons, including the well-described shortening of telomeres, but also because of DNA damage or the activation of oncogenes. Senescence is often posited to be a major driver of aging and diseases of aging, however, evidence in support of this view is scant. This is especially true in addressing the role of senescent cells in normal aging, which is perhaps experimentally intractable. Still, in the context of progeroid syndromes ("accelerated aging"), clearance of senescent cells in a mouse model does delay certain age-related tissue pathologies (Baker et al., 2011). Therapeutically speaking, however, it is

unclear whether deletion of senescent cells would have an overall beneficial effect on health, as it has been suggested (in keeping with the antagonistic pleiotropy theory of aging) that senescent cells have an anti-oncogenic effect early in life. In some sense, it is still mysterious why the activation of oncogenes would result in non-dividing but metabolically active cellular phenotype instead of simply death through apoptosis (Braig et al., 2005).

Mitochondria are also thought to play a central role in aging, especially in the context of oxidative stress. ATP production by mitochondria requires electrons to go through the mitochondrial electron transport chain (ETC), eventually producing H_20 and $C0_2$ through reduction of oxygen. Although this process is efficient, some of the 0_2 is not used up and thus becomes a free-radical capable of damaging DNA. Moreover, because mitochondria lack histones to protect its DNA, the mitochondrial DNA itself is highly susceptible to oxidative stress, creating a locally vicious cycle of damage and free-radical production. Evidence in support of this hypothesis includes the finding that knockdown of certain parts of the ETC results in greater longevity in fruit flies (Copeland et al., 2009) and that mutations in particular genes that confer longevity in worms results in decreased ETC function (Hekimi and Guarente, 2003). Again, however, whether free-radicals are a major mechanism of normal aging is not clear.

Towards a therapeutic?

Aging results in many cellular and tissue changes, and there are a host of potential mechanisms I have not touched on, such as the role of protein aggregation and homeostasis, autophagy, DNA repair pathways and sirtuins. But given all that we have learned over the last twenty years about the pathways implicated in aging, are we any closer to finding a Fountain of Youth in the form of a drug? Although Google recently founded Calico, a company dedicated to anti-aging research, the answer, unfortunately, appears to be not really.

Why have therapeutics against aging been so elusive? There are many potential reasons. As discussed earlier, and in line with antagonistic pleiotropy, many pathways that regulate longevity also have important roles in other vital functions. For example, though hydrogen peroxide is implicated as a free-radical in oxidative stress, it is also used in the cell as a signaling molecule. Thus, drugs modifying many of these pathways or molecules will have to achieve a kind of delicate balance that may pose a substantial challenge in terms of creating a beneficial therapeutic window. For example, the insulin signaling pathway discussed earlier may seem like an easy drug target, but dampening the pathway too much often results in a shortening of lifespan. In addition, not all tissues are created equal in their control over the aging process. Brain, muscle and fat, for instance, have all been shown to regulate certain aging processes at the

organismal level (how this is achieved remains a mystery). Thus, achieving tissue specificity will most likely be a desired, if not required, component of any drug against normal aging.

Perhaps, in the end, it will come down to what most of our doctors have been telling us all along: eat a little less, exercise a little more. For now, this is the unfortunate-for-the-lazy consensus derived from several decades of age-related research.

PART II: GOOD AXON, BAD AXON: REGENERATION IN THE NERVOUS SYSTEM

"The incapacity of the central nervous pathways to regenerate is a dogma accepted by science..."

- Ramon y Cajal

Introduction

The fact that axons of the peripheral nervous system (PNS) are capable of regenerating and reinnervating their downstream targets, whereas those of the central nervous system (CNS) generally are not, has remained both a formidable biological puzzle as well as the source of massive defects for patients suffering from brain or spinal cord injuries. Indeed, according to the National Institute of Neurological Disorders and Strokes, there are an estimated quarter of a million people living with spinal cord injury in the U.S. alone, along with nearly 800,000 strokes each year. Given this, advancing our understanding of what promotes neuronal regeneration is a critical scientific endeavor. Because the PNS is, in a sense, a mammalian model in which neuronal regeneration can be achieved, it represents a unique opportunity to understand mechanisms of regeneration in a mammalian system.

Historical work

Why do neurons of the CNS fail to regenerate axons? Broadly speaking, this could be due to either properties of the neurons themselves or the environment in which they reside, or both. Although distinguishing between these possibilities has been a fundamental goal of regeneration research, it is still a question that remains elusive today.

Early on it became apparent that the intrinsic growth of CNS neurons could be reactivated. In the beginning of the 20th century, Cajal described the work of his student Tello, in which he demonstrated that transplantation of sciatic nerve grafts into the cerebral cortex promoted regeneration in "white matter" (Cajal, 1991). This led Cajal to conclude that CNS neurons can regenerate if provided with a suitable environment. Many decades later, in the 1980s, these experiments were reproduced in a modern context by Agauyo and colleagues, who showed that regeneration could be obtained in the spinal cord if axons were permitted to regrow through a segment of sciatic nerve (Richardson et al., 1980). This work strengthened the idea that regeneration in the CNS was primarily an issue of location.

An inhibitory CNS

Subsequent work in the field focused on elucidating the inhibitory nature of the CNS environment. To this end, several inhibitory elements were identified in myelin, which is not adequately sequestered in the CNS, such as myelin-associated glycoprotein (MAG) (McKerracher et al., 1994), Nogo (Chen et al., 2000) and oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002). On the neuronal side, receptors that bind to these inhibitory ligands were also discovered, notably Nogo-Receptor (NgR) (Fournier et al., 2001) and Paired-immunoglobulin-like receptor B (PirB) (Atwal et al., 2008).

Another feature of CNS injury that does not occur within the PNS is the formation of a glial scar. This scar, which is thought to be composed largely of astrocytes as well as invading microglia and/or macrophages, creates a physical barrier that impedes axonal regeneration past the site of injury. Moreover, specific inhibitors of regeneration within the glial scar have also been identified, namely chondroitin sulfate proteoglycans (CSPGs) and keratan sulfate proteoglycans (KSPGs), (Silver and Miller, 2004). Likewise, the absence of receptors for these ligands on neurons, such as PTPsigma, promotes regeneration on inhibitory substrates (Shen et al., 2009).

Despite this knowledge, achieving substantial neurite outgrowth in the CNS, or functional recovery, has been limited when solely trying to overcome inhibitory

effects. For example, genetic deletion of the Nogo-receptor does not lead to increased neurite outgrowth (Zheng et al., 2005).

Neuronal intrinsic growth

The relative lack of success in promoting regeneration in the CNS through the disruption of inhibitory signals alone has spurred substantial investigation into the intrinsic growth potential of the neurons themselves. During development mammalian neurons grow, the local environment is permissive for such growth, and guidance cues are present that match the neurons with their specific targets, collectively leading to formation of functional neural circuits. In mature differentiated neurons the molecular machinery necessary for intrinsic growth is absent. However, injury to axons in the peripheral nervous system (PNS) increases intrinsic growth in sensory and motor neurons, and in optimal conditions, regeneration occurs, with restoration of function.

Improvement of intrinsic growth capacity has perhaps been most convincingly shown by “pre-conditioning” experiments in which a crush injury is performed on the nerve followed by a more proximal second crush. This pre-conditioning greatly boosts the rate of axonal regeneration (Forman et al., 1980). Pre-conditioning can also be demonstrated *in vitro*, where previously injured dorsal root ganglia (DRG) neurons have more robust neurite outgrowth than naïve neurons under permissive conditions **(see Chapter 2)**. Importantly from a

potential clinical standpoint, a pre-conditioning injury can also permit axons to overcome a normally inhibitory environment. For example, if the central branch (the axon within the dorsal column in the spinal cord that does not normally regenerate) of a DRG neuron is injured, along with an injury to the peripheral branch, the central branch can now regenerate into the spinal cord (Neumann and Woolf, 1999). Similarly, pre-conditioning is robustly recapitulated *in vitro,* where a nerve injury prior to culture of DRG sensory neurons results in massively increased axonal growth both under permissive and non-permissive conditions. How exactly pre-conditioning overcomes inhibitory environments is not well understood: does injury lead to the down-regulation of inhibitory receptors, or does it simply cause so much growth that it ignores any blocking signals?

In any case, the intrinsic growth capacity and pre-conditioning effect of PNS neurons is thought to be largely driven by the activation of transcriptional regulatory programs. Collectively, these programs are composed of "regeneration-associated genes (RAGs)" and several hundred of them are up or down-regulated after injury **(see Appendix)**.

Among these genes, many of them reproduce transcriptional programs utilized during development, suggesting common growth promoting mechanisms (e.g. GAP-43) (Benowitz and Routtenberg, 1997) while others (e.g. Sprr1a) are unique in their induction after peripheral nerve injury (Starkey et al., 2009). Importantly, it has been shown that cAMP levels increase in DRG neurons after a nerve injury

and this mechanism can allow central branches of DRG neurons to regenerate after dorsal column lesions (Qiu et al., 2002). In this sense, cAMP mimics pre-conditioning. In the search for transcription factors, several have been found. Notably, Activating Transcription Factor 3 (ATF3) enhances regeneration as ATF3-over-expressing mice regenerate their sensory axons more readily than wild-type mice after sciatic nerve injury (Seijffers et al., 2007). Likewise, c-Jun, which forms part of the AP-1 complex along with ATF-3, has also been demonstrated to be necessary for axonal regeneration (Raivich et al., 2004). In addition to these transcription factors, a number of other molecules have also been implicated in axonal regeneration in the PNS such as sterile 20-like kinases (Lorber et al., 2009) and heat-shock proteins (Benn et al., 2002; Ma et al., 2011). The precise mechanisms of how these molecules act, however, has not been elucidated.

Despite this progress, it is safe to say that the promise of recapitulating intrinsic growth potential of the PNS into the CNS has not yet been realized. No truly robust "master-regulator" of intrinsic growth has been identified, and future work should address the role of the entire network of RAGs rather than individual genes.

In the CNS, intrinsic growth programs are not induced by normal injury. For example, optic nerve crush in mice often leads to death rather than regrowth of retinal ganglion cells (RGCs) (Watkins et al., 2013). Still, recent work has

demonstrated the power of enhancing neuronal-intrinsic mechanisms in the CNS. Importantly, deletion of either PTEN or SOCS3 results in significant axonal regeneration in the optic nerve (Park et al., 2008; Smith et al., 2009). Moreover, double deletion of both PTEN and SOCS3 permits extraordinarily robust optic nerve regeneration (Sun et al., 2011). Interestingly, though the absence of these factors in RGCs clearly enhances intrinsic growth, it does not mimic the injured state of DRG neurons in the PNS, where both PTEN and SOC3 are expressed during regeneration (Sun et al., 2011). This highlights the potential differences in the transcriptional programs utilized to stimulate growth in these two different systems. Furthermore, it is interesting to speculate as to why there is an active regulatory presence of two genes that serve to seemingly constrain growth in these neurons. Would uninhibited growth of neurons in the CNS actually lead to damaging consequences? One could imagine, for instance, that evolution has favored the absence of regeneration in the CNS in order to prevent mis-wiring, leading to even more destructive consequences, or that adequately clearing the CNS environment through a Wallerian-like degeneration process might unnecessarily expose the CNS to the potential for autoimmunity.

Non-neuronal responses

Non-neuronal cells also play a critical role in axonal regeneration, one that is well described in the PNS and increasingly recognized in the CNS. Among non-neuronal cells, the best characterized in the context of regeneration are Schwann

cells and macrophages. One important role of these cells during regeneration is to clear debris from the distal stump of the nerve and make the site permissible for axonal regeneration. This process is referred to as Wallerian degeneration, and it is substantially slower in the CNS. During Wallerian degeneration, macrophages are recruited into the degenerating nerve and serve as a major pathway of phagocytosis of myelin and axonal-derived debris. Schwann cells also sequester and phagocytose myelin, a function that is particularly important at early time points after nerve injury **(see Chapter 2)**.

The role of macrophages has recently been underscored by studies showing that macrophages are necessary for axonal regeneration in the PNS. Impressively, Barrett et al. demonstrated that deletion of CD11b+ cells (macrophages, monocytes and granulocytes) completely abolishes neuronal regeneration (Barrette et al., 2008). Whether this result is simply due to efficient debris clearance or if macrophages directly promote regeneration via secretory factors or cell-cell/cell-axon interactions remains to be elucidated. Equally interesting has been work in the CNS showing that recruitment of innate immune cells can enable optical nerve regeneration (Yin et al., 2003; Yin et al., 2006). This is driven, at least in part, by oncomodulin, a neutrophil and/or macrophage derived factor capable of directly stimulating the growth of retinal ganglion cells (Kurimoto et al., 2013; Yin et al., 2006). Taken together, these studies suggest an important and broadening role for cells of the innate immune system in regeneration. In the future, it will be interesting to investigate other inflammatory cells, such as those

of the adaptive immune system, which are present for long periods after nerve injury and whose functions are completely unknown with regard to axonal regeneration.

Aside from sequestering and clearing myelin, Schwann cells in the distal stump that have lost contact with degenerated axons undergo de-differentiation and begin to proliferate rapidly (Mirsky and Jessen, 1996). This de-differentiation process, in which Schwann cells essentially trans-differentiate into non-myelinating "repair cells" is a key event in Wallerian degeneration. Recently, the transcription factor c-Jun was identified as a kind of "master-regulator" of Schwann cell de-differentiation. When c-Jun is conditionally ablated in the Schwann cell compartment, axonal regeneration in the PNS is severely limited (Arthur-Farraj et al., 2012). These results also serve to underscore the importance of Schwann cell activities in permitting optimal regeneration, a function often overlooked in the context of neuronal intrinsic growth.

Although c-Jun is clearly a major player in Schwann cell function, the molecular mechanisms that trigger, govern and maintain the de-differentiated Schwann cell state remain largely unexplored. Recent work has shown that specific activation of the ERK pathway in Schwann cells is sufficient to drive de-differentiation (Napoli et al., 2012). Interestingly, this work also demonstrated that c-Jun activation appeared to be downstream of ERK signaling. Other kinase pathways, however, are also capable of inducing de-differentiation, including both JNK

(Monje et al., 2010) and the p38 MAPK (Yang et al., 2012). Importantly, though, how Schwann cells sense injury and activate these kinase pathways is unknown, as is the question of how these kinase pathways interact or overlap to simultaneously enforce the repair cell phenotype while blocking the myelinating Schwann cell phenotype.

In their de-differentiated state, these repair cells form so-called “bands of Bungner” in the distal stump, creating a tunnel-like structure that is essentially a physically permissive environment for the regenerating axon. These bands also serve to permit subsequent re-myelination of new axons (Chen et al., 2000). Aside from these structural functions, Schwann cells also play a more direct role in promoting axonal regeneration by secreting neurotrophic factors that help to promote neuronal survival after injury (Koliatsos et al., 1993) and induce axon growth by initiating a signaling cascade that leads to the regulation of cytoskeleton binding proteins (Zhou et al., 2004). In addition, Schwann cells produce factors that form key components of the extra-cellular matrix (ECM), another part of the overall permissive PNS environment. One component of the ECM, laminin, is a permissive substrate *in vitro,* and likely a critical portion of the regeneration enabling environment *in vivo* (Luckenbill-Edds, 1997).

Future directions

Despite the somewhat prevailing belief that we have learned most of what there is to learn about regeneration in the PNS, there is still a substantial amount we do not yet understand, and by extension, much that we might learn about why regeneration does not proceed in the CNS.

As an example, we have only scratched the surface in terms of understanding how immune cells interact with neurons during regeneration. This is true for both the PNS, in which there is a well defined immune invasion of the sciatic nerve, but also the CNS, in which inducing inflammation with zymosan can promote significant axonal regeneration (Yin et al., 2003). At the level of the distal stump, we have thus far relegated immune cells to glorified vacuum cleaners, but given the depth and breadth of the inflammatory response (involving at a minimum monocytes, macrophages, neutrophils, NK, B- and T-cells) it seems highly likely they play unknown roles in axonal regeneration. Furthermore, the "non-immunological" role of immune cells is being increasing appreciated, where it was recently discovered that regulatory T cells actually help orchestrate regeneration in the muscle by directly secreting a factor capable of stimulating muscle stem cell differentiation (Burzyn et al., 2013). Given that regulatory T cells enter the distal stump shortly after nerve injury and persist there for at least thirty days (unpublished data not shown), it seems likely they may play a similar role in promoting regeneration in the PNS.

Given the breadth of tools available in immunology for enhancing and ablating various immune cells subsets (knockouts, depleting antibodies) the time is ripe for elucidating the roles of these cell-types. Using an anti-ly6G depleting antibody, we directly tested the contribution of neutrophils in promoting regeneration *in vivo*, and found no difference in functional recovery after injury in mice lacking these cells **(see Appendix)**. Interestingly, these results are in stark contrast with work done in the CNS demonstrating an important role for neutrophils in permitting axonal regeneration of the optic nerve (via secretion of the growth-promoting factor oncomodulin) (Kurimoto et al., 2013). Again, this suggests large differences in the mechanisms of CNS vs. PNS regeneration. If neutrophils are not necessary for axonal regeneration in the PNS, what role do they play considering their large numbers in the distal stump after nerve injury? Do neutrophils in the PNS secrete oncomodulin, and if so, are DRG neurons receptive to it in the same way that RGCs are?

Another major challenge in the field of peripheral regeneration has been to adequately distinguish the contributions of individual cell types. For example, much of what we have learned about neuronal intrinsic growth has come from transcriptomic studies of the whole DRG tissue. Although the DRG is certainly a decent surrogate for sensory neurons, it also contains glia, immune cells and fibroblasts. While the expression of individual genes within sensory neurons can be readily confirmed via in situ hybridization, in attempting to reconstruct entire genetic regulatory networks that may underlie regeneration-promoting programs,

this task becomes much more difficult, and by extension it is impossible to say whether certain transcriptional modules are a feature of neurons or non-neuronal cells. Thus, in the future, it will be worthwhile to elucidate the roles of non-neuronal cells within the DRG.

In a similar context, the field has tended to think of neuronal intrinsic growth as a predominately neuronal-driven event, for example, as a result of retrograde signals after injury (Rishal and Fainzilber, 2010). However, it seems plausible that signals derived during Wallerian degeneration, produced by either Schwann cells or immune cells, might also contribute to the activation of regeneration-associated gene-expression programs in neurons. To this end, Takao Omura and I observed a striking effect on *in vitro* pre-conditioning of DRG sensory neurons in the presence or absence of non-neuronal cells **(see Appendix)**. Although this does not necessarily reflect the more complex *in vivo* environment, it does suggest that non-neuronal cells, especially those contained with the DRG itself, have the capacity to modulate the intrinsic growth capacity of sensory neurons. This model intuitively make sense; perhaps non-neuronal cells in either the proximal stump or DRG react to injury by secreting factors that in turn promote the transcriptional activation of RAGs in sensory neurons. To put it succinctly, perhaps intrinsic growth is partially activated by extrinsic factors.

Finally, along these lines, I have noted, as have others, the strange correlation between the transcriptional programs activated after injury in the DRG and in the distal stump **(see Appendix)**. Among the transcripts up-regulated in Schwann

cells after nerve injury are c-Jun, ATF-3 and GAP-43, all important genes in the context of neuronal intrinsic growth. These data not only suggest caution in interpreting studies that assess the role of purported intrinsic growth genes through global knockout studies, but beg the question of why such a large degree of transcripts would be shared between these cells types. Though perhaps a bit farfetched for lack of evidence, it is interesting speculate as to whether this phenomenon may serve a functional purpose, such as controlling local protein synthesis in axons themselves (Court et al., 2008).

PART III: GREYING NEURONS: AGING IN THE PERIPHERAL NERVOUS SYSTEM

Aging and the PNS

While aging exerts its effects across all tissues, the nervous system is perhaps particularly vulnerable to age-related diseases and disorders. Although disorders involving the CNS, such as Alzheimer's, Dementia and Parkinson's are well appreciated from both a clinical and biological standpoint, age-associated changes in the PNS also strike the elderly frequently, and meaningfully contribute to a decreased quality of life.

One clear age-related change in the PNS is the gradual decline in nerve conduction velocity with increasing age. This decline has been demonstrated in a variety of species, including humans, and is associated with a host of physiological and structural changes within the nerves themselves (Adinolfi et al., 1991; Chase et al., 1992; Dorfman and Bosley, 1979; Verdu et al., 2000). Importantly, the amount of fiber in the nerve decreases with age. One study, for example, demonstrated in mice that nerves begin to show a rapid decrease in fiber after 20 months of age, with unmyelinated fibers being more vulnerable than myelinated ones (Verdu et al., 2000). Overall, reduced nerve conduction velocity in the elderly is likely the result of both shrinking axon diameter and reduction in myelination (Adinolfi et al., 1991; Chase et al., 1992). The reduction in

myelination is at least in part due do decreased expression of myelin-associated proteins such as P0 and MBP by Schwann cells (Melcangi et al., 1998). These data are supported by our microarray studies of the distal stump of aged animals, where we observe a decrease in the number of myelin-associated transcripts **(see Chapter 2)**.

Another broad physiological change of importance to the PNS is at the level of the neuromuscular junction (NMJ). A recent study demonstrated that after sciatic nerve transection, NMJ fragmentation and loss of motor end plate area were more severe in aged, but not young rats (Apel et al., 2009). It has also been suggested that the incidence of abnormal muscle reinnervation is greater in old mice after sciatic nerve crush (Kawabuchi et al., 1998).

Even in steady-state conditions, it has been nicely shown that NMJs of aged mice undergo significant structural alterations such as sprouting aberrations and fragmentation (Valdez et al., 2010). This represents one possible explanation for the motor defects and muscle atrophy well documented in aged animals. Interestingly, the same study demonstrated that caloric restriction and exercise are capable of attenuating or even reversing age-associated pathology (Valdez et al., 2010).

Importantly, it has also been demonstrated that the rate of axonal transport decreases with age, suggesting that the transport of essential factors to newly

regenerating axons be slower or compromised (Stromska and Ochs, 1982). In addition, this potentially makes maintenance of axons at a long distance from the cell body difficult. Finally, It has also been found that the expression of nerve growth factor receptors, such as TrkA and p75, decreases with aging (Parhad et al., 1995). This could lead to less uptake of growth factors, impairing cell health and regeneration. This in conjunction with the decreased expression of growth factors themselves from Schwann cells **(see Chapter 2)** may have doubly significant consequences on neuronal cell health.

These functional and structural changes in the PNS do result in clinical problems, even in the absence of nerve injury. Of particular significance in the aging PNS is small fiber neuropathy, which typically presents as pain in the feet in persons over the age of 60 (Lacomis, 2002). In general, peripheral neuropathies are frequent in the elderly, with some estimates suggesting that as high as 1 in 3 persons over the age of 65 suffer from some form of it, most of which are considered idiopathic in nature (Cho et al., 2006). Thus, understanding how the PNS ages should have important bearing on the treatment of neuropathies.

Aging and Peripheral Regeneration

The modern clinical study of peripheral nerve regeneration was, like so many other pressing scientific endeavors, born as a consequence of violence. During and after the first and second World Wars, when large numbers of soldiers

received peripheral nerve traumas from gunshot wounds and shrapnel injuries, it was observed that optimal functional recovery was rarely achieved. Fundamental work was then undertaken to determine basic information, such as the rate of axonal regeneration, and the factors that influence the course of recovery (Gutmann, 1942; Gutmann and Guttmann, 1942; Sunderland, 1952; Woodhall B, 1956). Although these early studies often speculated about the age of the patient as a clinical factor, the relatively age-homogenous population of soldiers prevented any clear correlations from being drawn.

Still, contemporary clinical data continues to indicate that age is an important predictor of the functional outcome following peripheral nerve injury (Lundborg and Rosen, 2001; Nagano, 1998). Indeed, that age impairs axonal regeneration after nerve injury appears to be a conserved process, being observed in mice, rats, zebra fish (Vaughan, 1992; Verdu et al., 2000; Wada et al., 2013) and even the nematode worm C. *elegans* **(see Appendix)**. Despite this widespread finding, the mechanisms underlying this decline have remained largely unexplored.

One notable early study used histological based methods (mostly electron microscopy) to show that Wallerian degeneration, myelin clearance and the appearance of newly regenerating axons were all slower in rats after facial nerve crush (Vaughan, 1992). The same study also found that aged rats regrew fewer total axons after injury. In the same vein, other studies have used histological

methods in mice to suggest that aged animals regenerate fewer myelinated axons after sciatic nerve injury (Tanaka and Webster, 1991; Tanaka et al., 1992). Although functional recovery has not been directly assessed in animal models, one report did note an observational delay in the resumption of "whisking behavior" after facial nerve injury to aged rats (Vaughan, 1992).

The role of aging non-neuronal cells in peripheral nerve regeneration has also not been addressed. As Wallerian degeneration appears to be delayed in aged animals (Tanaka and Webster, 1991; Vaughan, 1992), it has been suggested that Schwann cell and macrophage responses may diminish with aging, but these results could equally be the product of axonal-protective mechanisms. While scarce, direct evidence of Schwann cell dysfunction with normal aging includes a reduction of Schwann cell proliferation in the nerve after injury (Komiyama and Suzuki, 1991), as well as reduced proliferation *in vitro* (Komiyama and Suzuki, 1992). More recently, a large gene-expression survey found similarities with steady-state Schwann cells in aged animals and the expression signatures of disease models of demyelinating diseases, predominantly finding differences in lipid metabolism and expression of immune-related genes (Verdier et al., 2012). Finally, a paper using time-lapse imaging of axonal regeneration in mice *in vivo* showed that the rate of regeneration in aged animals was unaltered, but ran into "road blocks" that likely represented Schwann cells (Kang and Lichtman, 2013). Taken together, surprisingly little is

known about the mechanisms leading to decreased neuronal regeneration in aging.

CHAPTER TWO

DIMINISHED SCHWANN CELL REPAIR RESPONSES UNDERLIE AGE-ASSOCIATED IMPAIRMENT IN AXONAL REGENERATION

Forward

This Chapter is a slightly modified version of a manuscript currently being resubmitted to *Neuron* after revisions to deal with reviewer comments. While I have participated in several projects during the course of my PhD, understanding how the peripheral nervous system ages, and in particular, why neuronal regeneration is impaired as a result of aging, has been the central topic of my thesis work.

Contributions

The overall idea for this project came as a kind of marriage between two of my rotations, one with Amy Wagers and the other with Clifford Woolf. In Amy's lab I learned about aging and strategies for rejuvenating aging such as through parabiosis. An obvious question to ask upon joining Clifford's lab, then, was how does aging affect axonal regeneration, a key topic of study in the Woolf Lab. Subsequent experimental design was carried out by myself, Clifford and also in conjunction with Takao Omura, who served as my "day-to-day" advisor in the lab. The paper itself was written primarily by myself and by Clifford, with meaningful contribution from Amy Wagers.

Technically speaking, although I directly participated in every experiment included in this version of the manuscript, I of course had a great deal of help,

from both members of my lab and outside. In particular, I frequently worked alongside Takao Omura, who showed me most of the basic skills in the lab, such as sciatic nerve crush and DRG dissection and culture, and helped tremendously in getting early experiments up and running quickly, including performing sciatic nerve crush on the first cohort of animals and often working in parallel with me. Takao's technical expertise was also instrumental for several experiments, and without him surely they would not have been accomplished: the nerve grafts surgeries could only have been from his hands. Amanda Brosius-Lutz in the Barres lab at Stanford made essential contributions in both the design and execution of key Schwann cell experiments, including the imaging and quantification of myelin, macrophage influx into the nerve and Schwann cell de-differentiation. Inge Cheng performed key c-Jun Western Blot analyses. Christine Miller was the point person in performing parabiosis surgeries. Others listed as authors also made either intellectual or technical contributions to this manuscript.

Overall, it has been a great pleasure of my PhD to be able to work with so many wonderful collaborators, and I certainly would not have accomplished this thesis without their help, and they deserve immense credit.

Diminished Schwann cell repair responses underlie age-associated impairment in axonal regeneration

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RUNNING TITLE

Rejuvenating axonal regeneration in aged mice.

SUMMARY

The regenerative capacity of the peripheral nervous system (PNS) declines with age. Why this occurs and whether it can be reversed, however, remains unknown. Here, we demonstrate that 24-month old mice exhibit an impairment of functional recovery after nerve injury compared to 2-month old animals. Surprisingly, we find no difference in the intrinsic growth capacity between aged and young sensory neurons *in vitro*, nor in their ability to activate growth-associated transcriptional programs after injury. Instead, functional recovery in aged animals can be fully restored by transplantation of young nerve grafts. **Conversely, aged nerve grafts diminish axonal regeneration in young hosts.** Molecular interrogation of the sciatic nerve reveals that aged Schwann cells (SCs) fail to efficiently and rapidly activate a transcriptional repair program after injury, including a delay in the up-regulation of c-Jun protein. Functionally, aged SCs exhibit impaired de-differentiation and myelin phagocytosis. These results suggest that the age-associated decline in axonal regeneration results from diminished Schwann cell plasticity after denervation.

INTRODUCTION

Successful regeneration of the PNS requires the coordinated activity of several distinct yet interlocking systems. After axonal injury, neurons activate transcriptional "intrinsic growth" programs and begin extending new axons. Schwann cells (SCs) in the distal nerve undergo a rapid reprogramming process, de-differentiating into non-myelinating repair cells that secrete pro-regenerative factors, uptake non-permissive myelin, recruit macrophages and create physical tracks onto which newly growing axons can navigate. Macrophages, in turn, enter the site of nerve injury from the circulation and help to clear myelin and axonal-derived debris, obstacles to regrowth. Remarkably, these complex intrinsic and extrinsic processes can lead to restoration of function after peripheral nerve trauma.

As with most mammalian tissues, however, the regenerative capacity of the PNS declines with advancing age (Nagano, 1998; Pestronk et al., 1980; Tanaka and Webster, 1991; Vaughan, 1992; Verdu et al., 2000; Woodhall B, 1956). This decline has been recognized in humans since at least World War II, when follow-up studies of patients suffering from peripheral nerve traumas demonstrated a correlation between age and the extent of functional recovery (Woodhall B, 1956). This correlation continues to be observed in clinics today, such as in the case of brachial plexus injuries (Nagano, 1998). In addition, broader defects of the aging PNS have also been widely noted. For example, it is estimated that

nearly 1 in 3 persons over the age of 65 suffer from some form of peripheral neuropathy, and the underlying cause in the majority of these instances is considered idiopathic (Cho et al., 2006). Thus, understanding how the PNS changes with normal aging is of significant clinical importance.

To some degree, these clinical associations between age and the extent of neuronal regeneration have been recapitulated in animal models. While not addressing functional recovery directly, early work demonstrated an age-associated impairment of axonal regeneration in both mice and rats through histological assessments (Verdu et al., 2000). More recently, the developmental decline in axonal regeneration that is found in the nematode worm C*. elegans* was shown to be the result of neuronal-intrinsic alterations (Zou et al., 2013)**.** In the case of mammals, however, to what degree age-associated impairment of axonal regeneration represents neuronal-intrinsic alterations versus environmental perturbations remains an open and critical question, with both mechanisms having been previously speculated about and with significantly different therapeutic implications.

In this study, we use 24-month and 2-month old mice along with standard models of peripheral nerve injury to assess the potential contributions of neurons, immune cells and peripheral glia to the aging phenotype. Surprisingly, our results demonstrate that extrinsic factors are sufficient to rejuvenate the age-associated

decline in mammalian axonal regeneration, and broadly reveal how Schwann cell function and its response to axonal injury alters during normal aging.

RESULTS

Although previous studies have established in rodents that fewer axons regenerate after injury in aged animals, it is unclear how this might translate into functional recovery, a meaningful clinical parameter. We thus first sought to establish whether aging impaired functional recovery in mice after nerve injury. To accomplish this, we performed a sciatic nerve crush injury (a model in which full recovery is expected (Ma et al., 2011)) on cohorts of 2, 12, and 24-month old C57BL/6 mice, and assayed their sensory and motor functional recovery over time, using behavioral-based assays (see methods). In young animals, full sensory recovery is achieved approximately 15 days after injury, while motor function is restored by day 30. Aging, however, produces a progressive defect in the onset and time to restoration of motor and sensory function, with an approximately 3-4 day difference in full recovery between young and aged animals for sensory function and a 9-10 day delay for recovery of motor function **(Figures 2.1A, 2.1B, 2.2A, 2.2B)**. Middle-aged animals (12-month) show an intermediary phenotype, indicating that age-dependent slowing of functional recovery is acquired gradually throughout life.

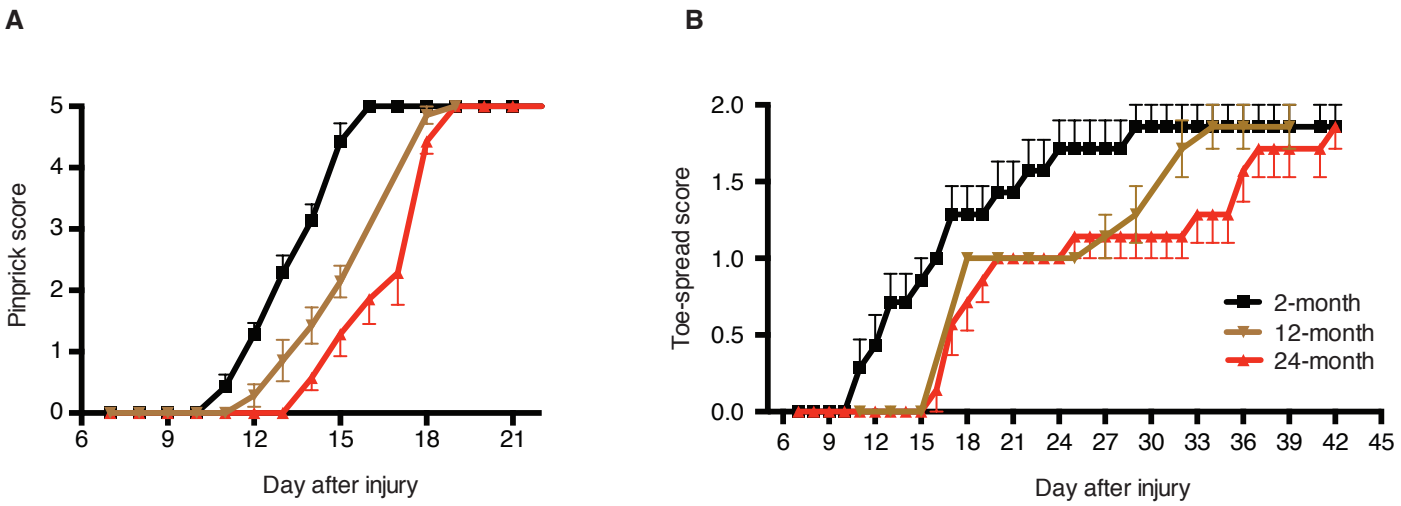

Figure 2.1: Progressive, age-dependent deficit in peripheral nerve regeneration. Cohorts of 2, 12 and 24-month old mice were subjected to sciatic nerve crush injury and restoration of sensory (A) and motor (B) function, assayed through pinprick and toe-spreading assays, respectively. Mean ± SEM, n = 7-8 mice per cohort.

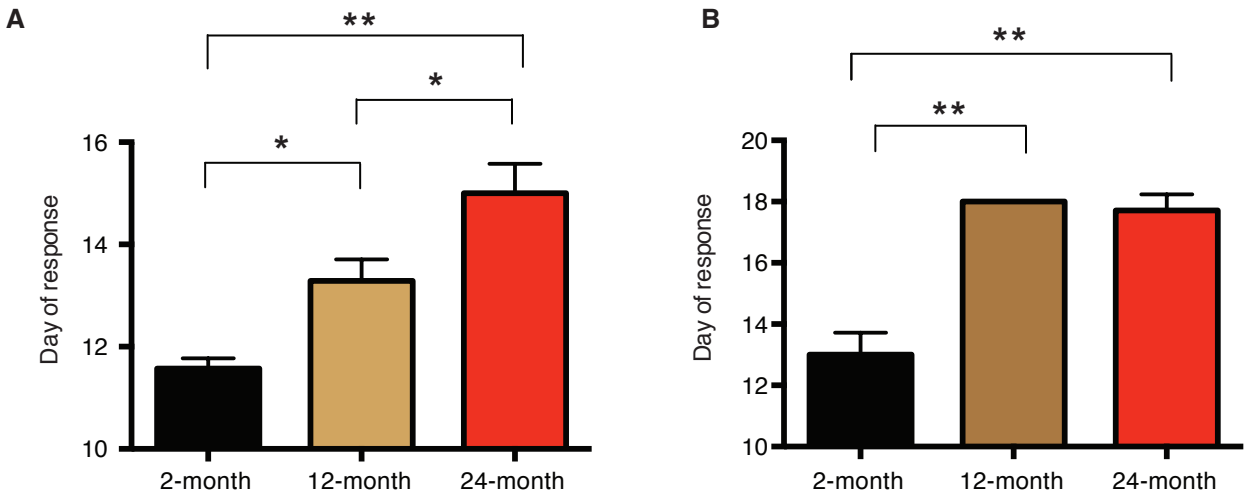

Figure 2.2: Age-dependent delay in functional recovery after nerve injury. Cohorts of 2, 12 and 24-month old mice from were subjected to sciatic nerve crush injury and the first day in which a positive sensory (A) or motor (B) behavioral response was recorded. *P < 0.05; **P < 0.01 in one-way ANOVA with Bonferroni's multiple comparisons test.

What is responsible for this defect? Because axonal regeneration in mammals is driven by the transcriptional activation of neuronal "intrinsic growth" programs after injury (Chen et al., 2007; Seijffers et al., 2007), it has been widely assumed that the age-related decline in regeneration is the consequence of a diminished intrinsic growth potential of aged neurons (Pestronk et al., 1980). Unexpectedly, however, transcriptional profiling from young (2-month) and aged (24-month) dorsal root ganglia (DRGs), both before and after nerve injury, revealed extremely comparable gene-expression signatures, with similar levels of injury-induced transcriptional activation between young and old ages **(Figures 2.3A, 2.3B, 2.4A)**. After nerve injury, over 250 genes in the DRG are up or down-regulated greater than 2-Fold compared to naive controls (**Figure 2.4B)**. Among these, only 14 are differentially expressed (> 2-Fold) between young and aged animals. Many of these age-variant transcripts are also de-regulated in naive (uninjured) conditions, implying they are part of a "background" aging signature and not specific to nerve injury **(Table S1)**. Important "regeneration-associated genes" such as ATF3, GAP43, and Sprr1a, were not differentially expressed between young and aged DRGs **(Figure 2.3B)** (Bonilla et al., 2002; Chong et al., 1994; Seijffers et al., 2007). As predicted from the transcriptional data, *in vitro* neurite outgrowth assays revealed comparable growth potential regardless of age in both naive and pre-conditioned DRG sensory neurons, confirming no deficit with age in the intrinsic neuronal growth induced by injury **(Figure 2.3C-G)**. These experiments strongly argue that a mechanism extrinsic to neurons influences the regenerative potential of aging DRG neurons *in vivo*.

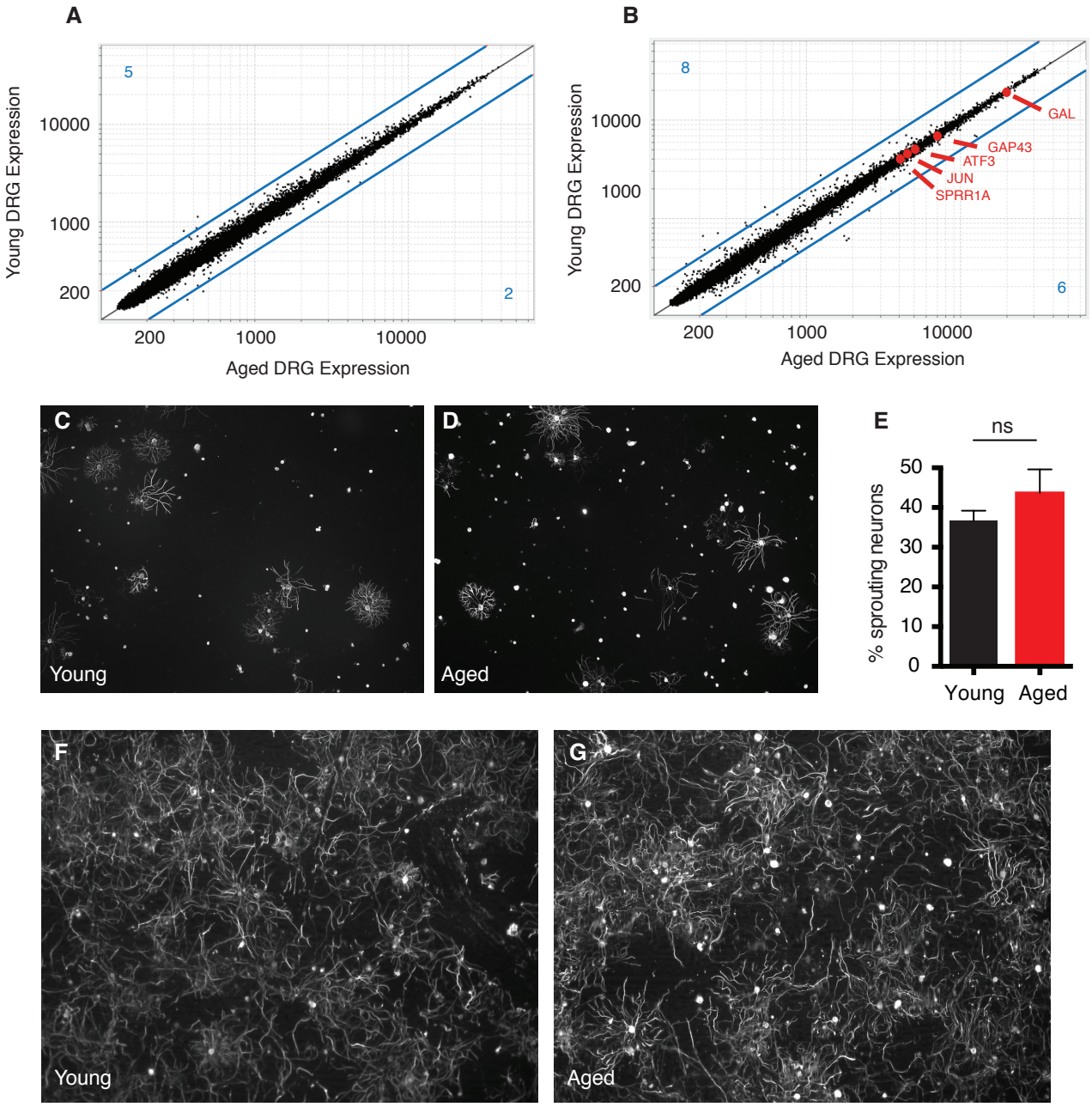

Figure 2.3: Neuronal intrinsic growth responses to injury remain robust throughout life. (A and B) Microarray data from DRG neurons. Expression vs. expression of young vs. aged DRGs in (A) naive conditions and (B) 4 days after nerve crush injury. Known markers of regeneration are highlighted in red. Lines in blue represent Fold Change = 2, blue numbers in each corner represent the number of genes over- or under-expressed by more than 2-Fold. (C to G) In vitro neurite outgrowth assays of DRG sensory neurons. Sensory neurons from naive young (C) or aged (D) DRGs from aged or young animals were purified and cultured for 17 hours and neurite outgrowth measured with Neuromath (E). Mean ± SEM, n = 3 independent experiments per group, NS = not significant with unpaired t-test, Welsh's correction, two-tailed. DRG sensory neurons from young (F) or aged (G) animals were purified 4 days after nerve injury, and cultured for 12 hours, revealing robust pre-conditioning responses.

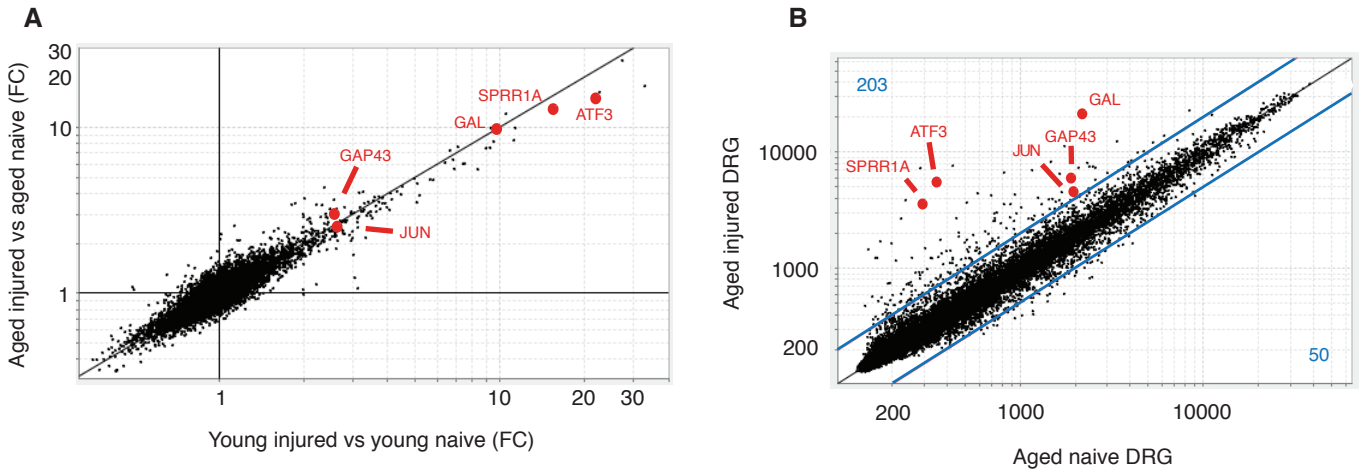

Figure 2.4: Transcriptional profiles of young and aged DRGs are comparable.
Gene-expression data of young and aged DRG neurons. (A) Fold Change vs. Fold Change plot. Genes along the black y=x line represent equal Fold Change between naive and injured conditions of either age. Note the vast majority of genes fall roughly along this line. Several genes along the y=1 line that are seem to be activated only in young animals represent spurious muscle contamination (MYL1, TNNI2, ATP2A1, TNNC2, ACTA1) with t-test p-values well above 0.4. Red = known regeneration-associated genes. (B) Expression vs. expression plot of aged naive vs. aged injured DRGs. Note the injury response is robust. Several known regeneration markers are highlighted in red. Blue lines repr-sent Fold Change = 2, blue numbers in each corner represent the number of genes up or down-regulated by Fold Change of 2.4

As such, we hypothesized that allowing "aged" axons to regenerate in a "youthful" environment might enhance functional recovery in aged animals. Conversely, we conjectured that forcing young axons to regenerate in an "aged" environment would result in impaired recovery. To test this, we harvested 1 cm segments of sciatic nerve from either aged or young animals and directly sutured them into the sciatic nerves of either old or young animals. In this way, axons from the host animal must regenerate through a 1 cm segment of either age-matched or age-mismatched nerve tissue before reaching their targets in the skin **(Figure 2.5A)**. We assessed sensory functional recovery to monitor regeneration. Because transection/re-suture injuries are more severe than crush injuries, motor recovery of plantar muscles in the foot is not obtained, and functional sensory recovery takes much longer (Ma et al., 2011). Strikingly, we observed that functional recovery was enhanced in aged animals receiving young nerve grafts such that it matched young animals receiving young nerve grafts **(Figure 2.5B)**. Reciprocally, young animals receiving aged nerve grafts showed slower functional recovery, similar to aged animals receiving aged nerve grafts **(Figure 2.5B)**.

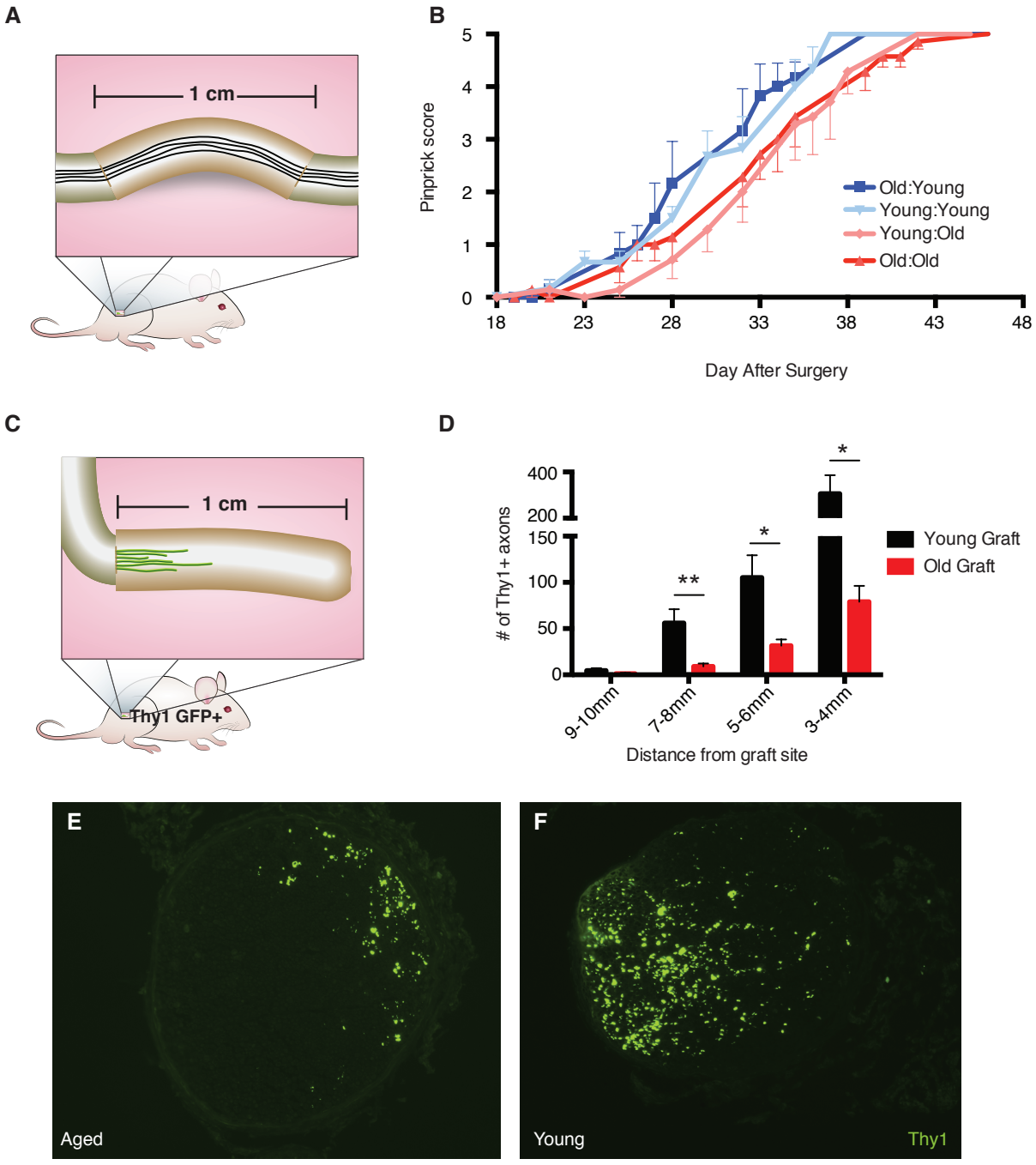

Figure 2.5: Neuronal-extrinsic factors are sufficient to recapitulate or rejuvenate the phenotype. (A) Schematic of nerve graft strategy. 1 cm long segments from young or aged animals were sutured into the nerves of either young or aged host animals and restoration of sensory recovery assayed as in Fig. 1. (B) Time-course of sensory recovery as measured by pinprick score. (C) Schematic of Thy1 YFP+ graft strategy. 1 cm long segments of sciatic nerves from either aged or young mice were sutured onto the sciatic nerves of young Thy1 YFP+ host animals and nerves analyzed histologically after 7 days (D). Representative images of YFP+ axons in the donor aged and young nerve grafts, 5mm distal to the site of suture. (E) Quantification of the number of YFP+ fibers at each specified distance from the suture site. Axons counts were quantified using ImageJ. Mean ± SEM is plotted, n = 7 mice per group (one mouse with counts greater than 3 SD from the mean was removed from the dataset), *P <0.05, **P <0.01 with unpaired t-test, Holm-Sidak correction.

We further quantified the extent of axonal regeneration in an age-mismatched system. To accomplish this, we used a nerve graft strategy in which either young or old 1 cm long pieces of sciatic nerve were sutured onto the nerves of Thy1 YFP+ young host animals (aged transgenic mice were not available for the reciprocal experiment) (**Figure 2.5C)**. Thy1 YFP+ mice were chosen to enable visualization of regenerating fibers in the graft. After one week, the number of YFP+ axons growing into either young or aged donor tissue was quantified histologically. Forcing young axons to regenerate into an aged environment, as opposed to a young one, severely impaired the extent and length of growth with reduced numbers of axons at every distance from the site of the graft **(Figure 2.5D and 2.5E)**.

We next sought to identify what age-associated changes occur within the sciatic nerve environment. Transcriptional profiling of young and aged nerves distal to a sciatic nerve ligation (to prevent regenerating axons from contaminating samples) revealed widespread age-related alterations (**Figure 2.6A, 2.7A, 2.7B, Table S2, Table S3)**. This can be seen most prominently by the number of transcripts that deviate from the Y=X parity line in the fold change versus fold change plot, indicating that hundreds of genes up- or down-regulated after injury in the young are not equally regulated in aged nerves. Strikingly, we found that many of these genes are associated with a "repair program" in Schwann cells (SCs) activated after injury **(Figure 2.6A, Table S3)**. After nerve injury, SCs undergo a phenotypic reprogramming process whereby they down-regulate their

myelinating phenotype, rapidly divide, and acquire a non-myelinating, "repair cell" phenotype critical for the development of a growth permissive environment within the nerve that facilitates regeneration (Arthur-Farraj et al., 2012; Mirsky et al., 2008).

Figure 2.6: Acquisition of repair cell phenotype is impaired in aged Schwann cells.

(**A)** Expression data from the distal nerve stump of aged and young animals after nerve transection/ligation injury. X axis represents Fold Change of young injured against young naive transcripts. Y axis is Fold Change of aged injured against aged naive transcripts. Transcripts along the black y=x line represent equal up or down regulation after injury compared to naive. Red = myelin-associated genes; Blue=growth factors-associated; Orange=mitosis-associated. (**B)** Representative Western blot analysis of total c-Jun. Cross-sections and whole mounts of sciatic nerves from aged and young animals at day 0, 3, 5 and 7 after nerve crush injury stained with (**C)** S100 and p75, **(E)** MBP, and **(G)** CD68. **(D, F and H)** Quantification of staining from (**C, E and G)**. Mean ± SEM is plotted, n = 3-4 mice per group, *P < 0.05, **P < 0.01 with unpaired t-test, Holm-Sidak correction.

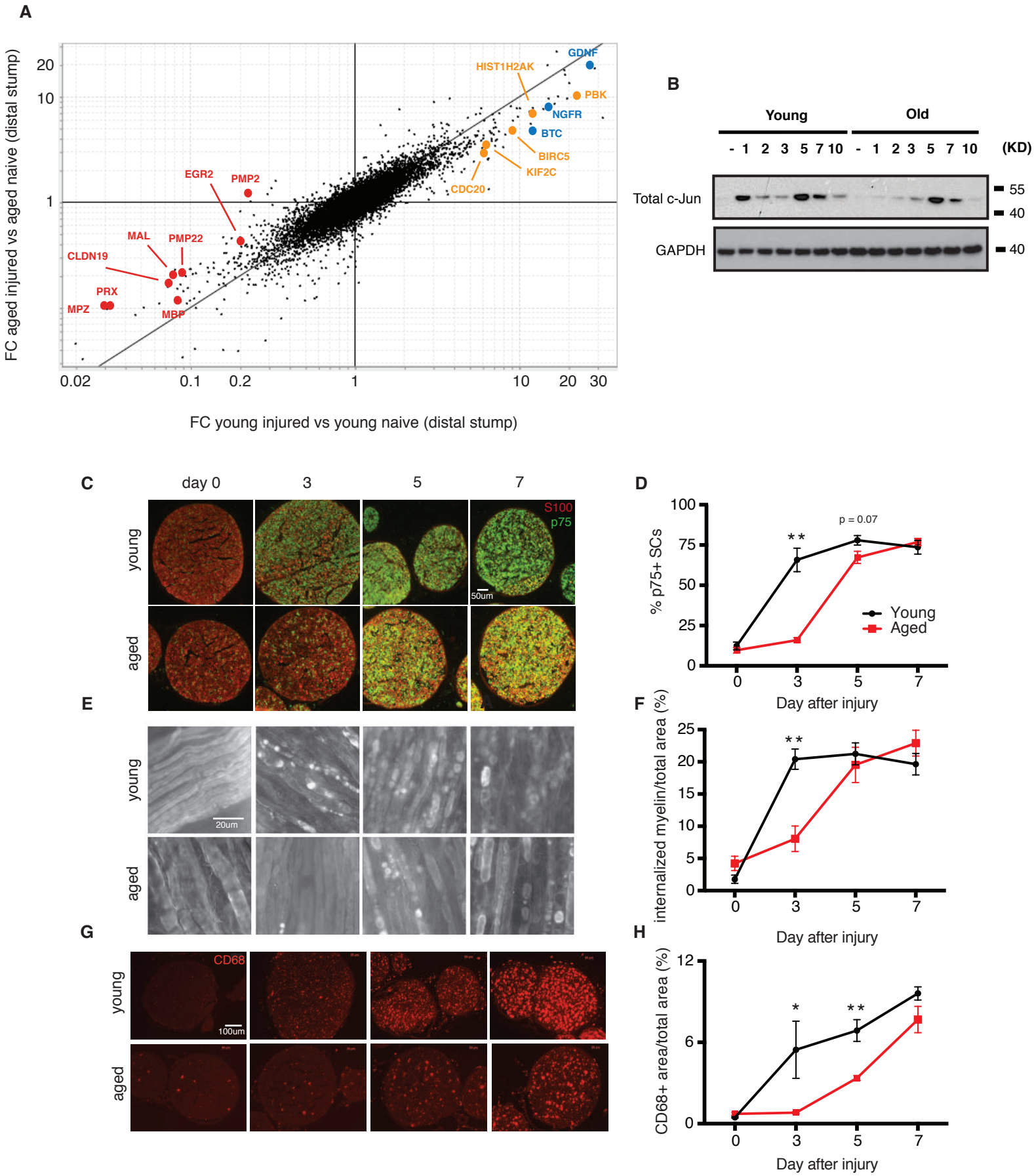

Figure 2.6 (cont): Acquisition of repair cell phenotype is impaired in aged Schwann cells

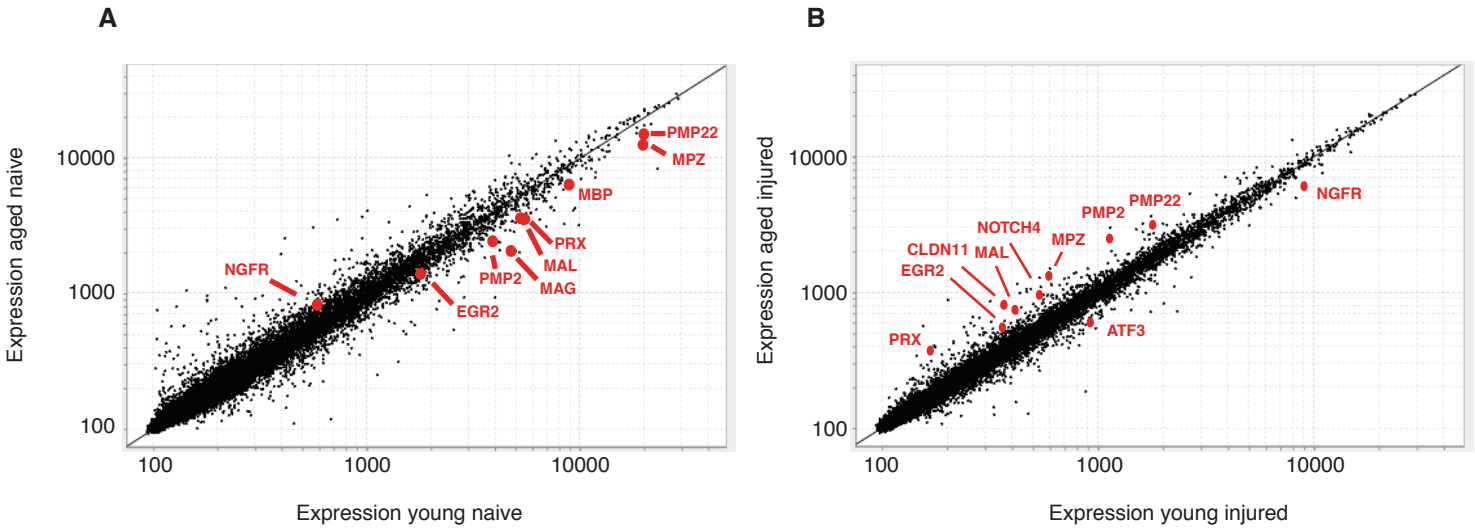

Figure 2.7: Transcriptional alterations in aged Schwann cells.
(A) Expression vs. expression of naive aged vs. young nerves is plotted. In red, several SC-associated transcripts are highlighted. Note the expression of myelin-related genes is down-regulated in aged animals under steady state conditions. (B) Expression vs. expression of injured aged vs. young nerves (day 4 after sciatic nerve crush). Select SC-associated genes are highlighted in red.

We observed a prominent age-related deregulation of genes encoding key repair cell functions. Genes down-regulated in aged nerves relative to young ones after injury included those associated with growth factors (BTC, NGFR, BDNF) and mitosis (e.g. KIF2C, PBK, BIRC5, CDC20) **(Figure 2.6A, Table S3)**. Likewise, genes associated with myelin production (e.g. PMP2, MPZ, MAL, EGR2) were relatively over-expressed in lesioned aged animals, implying an impaired ability to down-regulate their myelinating phenotype after injury **(Figure 2.6A)**.

Recently, the transcription factor c-Jun was identified as a "master regulator" of the SC repair phenotype after nerve injury, and SC-specific ablation of c-Jun results in severely impaired functional recovery (Arthur-Farraj et al., 2012). Interestingly, we noticed marked similarities between the transcriptional profiles of injured aged nerves and genes altered in mice lacking c-Jun expression in SCs after injury (e.g. MPZ, CDH1, SEMA4F) suggesting that genes under control of c-Jun were altered in aged animals (Arthur-Farraj et al., 2012). Strikingly, Western blot analysis demonstrated that the regulation of c-Jun protein was significantly altered in the nerves of aged animals immediately after injury **(Figures 2.6B and 2.8)**. This initial burst of c-Jun expression in the nerves of young animals 1 day after injury was approximately 5-fold higher than in aged animals **(Figure 2.8)**. ERK and JNK, two upstream kinase pathways of c-Jun, were unaltered in aged sciatic nerves after injury **(Figure 2.9)**.

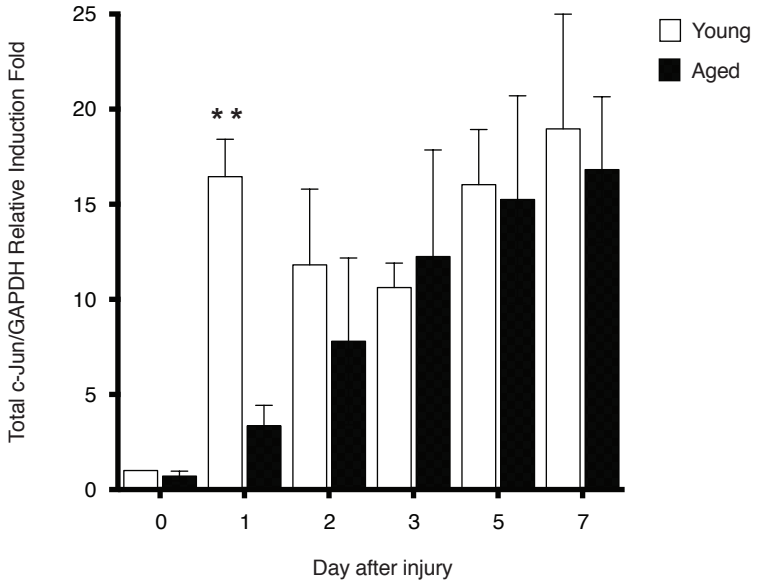

Figure 2.8: Early c-Jun deregulation in aged nerves after injury. (A) Summary of Western blot analyses of c-Jun regulation in young and aged nerves (distal to the site of injury) after nerve injury. n = 3 biological replicates for all time-points except day 7, where n = 2. **P < 0.01 with unpaired t-test, Holm-Sidak correction.

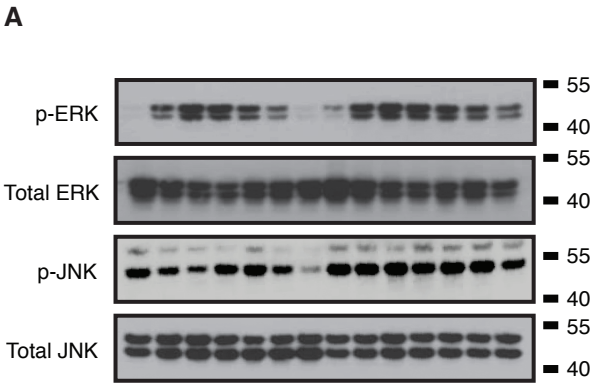

Figure 2.9: ERK and JNK regulation unaltered in aged nerves. (A) Representative Western blot analysis of described proteins.

Given that aged SCs are unable to activate transcriptional repair programs after nerve injury, one would predict functional consequences in the SCs, and more generally, for Wallerian degeneration in the nerve. As predicted, expression of p75, a marker for the repair cell phenotype was delayed in aged SCs after sciatic nerve crush injury compared to young controls. In young animals, ~65% of S100+ SCs were positive for p75 3 days after nerve injury (**Figures 2.6C and 2.6D)**. S100 is a SC marker present in intact and dedifferentiated SCs. In aged animals, however, p75 reactivity in only ~16% of S100+ SCs was observed.

A key downstream function of SCs after injury is the intracellular accumulation and sequestration of myelin from their cell membrane. Myelin is an inhibitory substrate for axon growth, and it is thus essential for external myelin to be cleared from the distal injury site before regeneration can occur (McKerracher et al., 1994). SCs from aged mice showed a deficit in their ability to sequester and uptake myelin during early time points after injury, in parallel with the delayed expression of p75 **(Figures 2.6E and 2.6F)**. Dense MBP positive myelin accumulations were localized within p75 positive (dedifferentiated) SCs soon after injury in young nerves but not in p75 positive aged SCs. The defect can be clearly seen from myelin staining in distal segments of the sciatic nerve after crush injury, where 3 days after injury SCs in young nerves show bright and punctate myelin staining, indicating that myelin is being internalized and cleared, while in aged animals myelin staining is diffuse along axon segments as in intact nerves.

Macrophages are required for axonal regeneration to remove axonal debris as well as any remaining myelin not removed by SCs, and macrophage recruitment into the degenerating nerve is dependent upon factors secreted by de-differentiated SCs (Barrette et al., 2008; Napoli et al., 2012). We thus measured macrophage recruitment into the degenerating nerve by tracking the expression of CD68, a standard marker for activated macrophages, over time. This revealed a markedly delayed recruitment of CD68+ macrophages for up to 7 days after injury in aged animals **(Figures 2.6G and 2.6H)**.

In addition, we also wondered whether restoration of "young" circulating factors in aged animals might partially reverse the diminished functional recovery observed in 24-month old animals. To address this question, we performed heterochronic parabiosis, in which aged and young animals are surgically conjoined such that their circulatory systems are shared. This method was recently used to successfully reverse an age-associated defect in re-myelination of the nervous system driven by macrophage dysfunction (Ruckh et al., 2012). After thirty days of parabiosis to allow for proper chimerism between animals we performed sciatic nerve crush injury on the aged partner and followed the restoration of sensory and motor function every day for ~45 days using behavioral assays as earlier **(Fig 1).** Unfortunately, all old:old control pairs died before the completion of the experiment. As shown in **Figures 2.10A-2.10D**, however, exposure of "youthful" systemic factors did not affect the regenerative phenotype of aged animals, with the difference in recovery rates being very

comparable to aged and young animals in the absence of parabiosis **(Fig. 1).** Again, as parabiosis has been demonstrated to rejuvenate age-associated defects in a variety of paradigms these results were not expected and suggested to us that intrinsic defects in circulating factors were not likely to be driving age-related defects in regeneration.

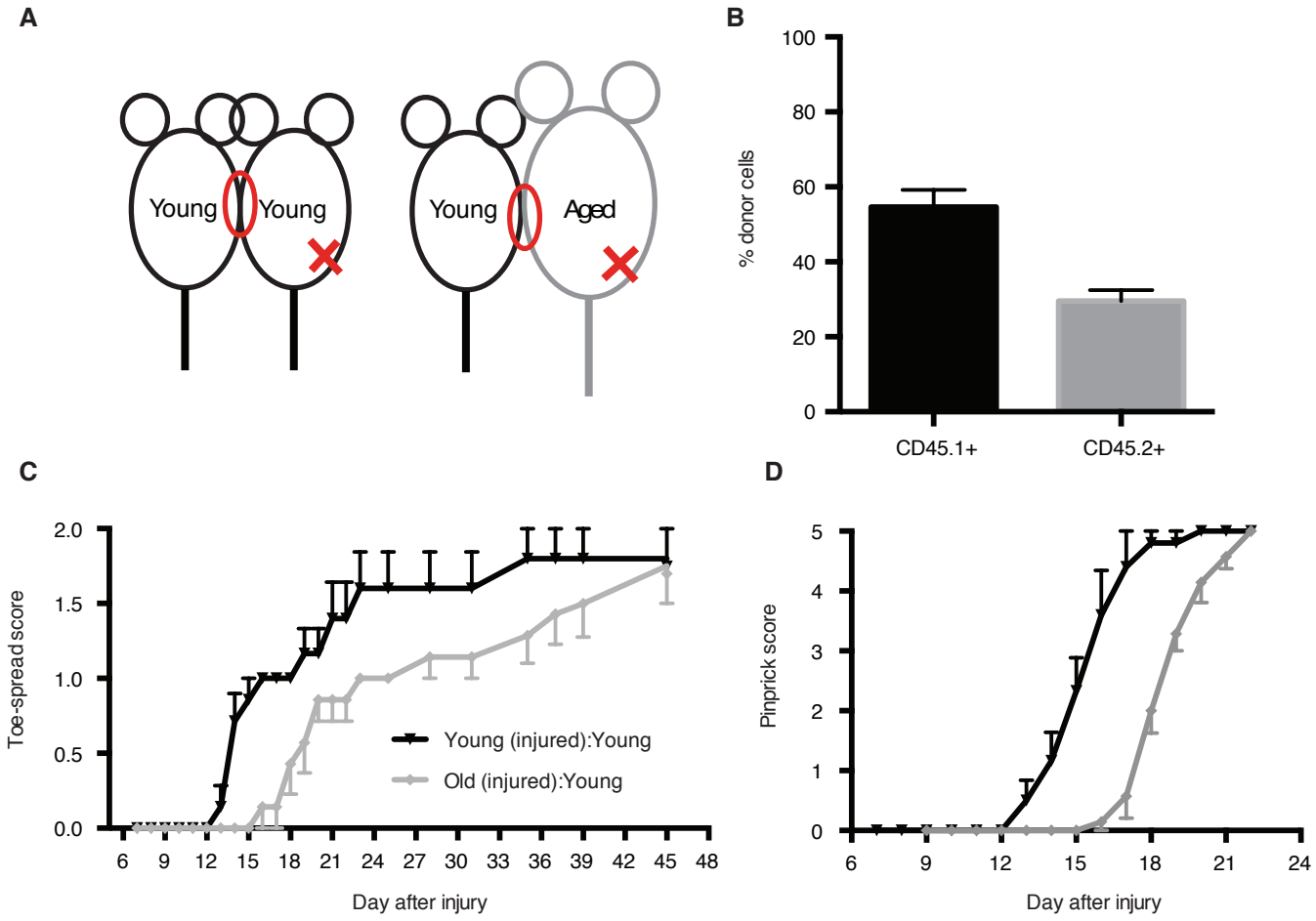

Figure 2.10: Parabiosis does not affect regeneration in aged animals.
(A) For parabiosis experiments, congenic mouse strains were used (one partner CD45.1+ and one partner CD45.2+). Chimerism of blood cells was thus evaluated by flow cytometry after parabiosis by evaluating the percentage of donor blood within spleens of the host animal. Plots are presented as mean ± SEM, n = 4 mice for each congenic strain. (B) Schematic of parabiosis experiment. Young:young and aged:young pairs were surgically conjoined for thirty days to allow for chimerism and then subjected to sciatic nerve crush. Restoration of functional recovery in the injured partner was assessed through pinprick (C) and toe-spreading (D) as in Fig. 1. Mean ± SEM, n = 7 per cohort to begin (1 young:young pair died at day 17, another died at day 21, 1 old:young pair died at day 39, 2 others at day 45).

DISCUSSION

Our work presents several interesting findings. First, we show that aging introduces a progressive decline in axonal regeneration after injury in mammals, akin to work done in C*. elegans* and mirroring human clinical data. Given that in mice, as in humans, a critical period is observed in which functional motor recovery will not be obtained without timely target re-innervation (Ma et al., 2011), this slowing in regenerative potential suggests an age-dependent mechanism that may contribute to suboptimal clinical outcomes after nerve trauma in elderly individuals.

Furthermore, our data also demonstrate that the regenerative capacity of the aging mammalian PNS is not limited by neuronal responses as previously assumed, but is profoundly influenced by extrinsic factors emanating from the surrounding tissue. Unexpectedly, we found that neuronal intrinsic growth responses are unaltered with normal aging, indicating that aged neurons can be fully "primed" to regenerate after an axonal injury. We further demonstrated that the ectopic introduction of aged tissue could impede regeneration in young, healthy mice, whereas grafting of young tissue liberated aged neurons to express their full, robust repair potential. These observations indicate that deficient axonal regeneration in aged animals arises from dominant, extrinsic inhibition of neuronal outgrowth by the aged microenvironment and suggest that functional

recovery after axotomy may be enhanced throughout life via neuron-extrinsic mechanisms.

Our analysis demonstrates that SC injury responses are substantially altered in aged animals, suggesting a failure of aged SCs to effectively activate an appropriate transcriptional repair response. These data implicate aged SCs as key mediators of age-dependent inhibition of axon regeneration in the PNS. Although it remains to be determined whether the altered SCs responses of aged animals result entirely from intrinsic defects (age-acquired DNA damage, e.g.) or whether this represents a failure in an upstream signaling cascade (e.g. leading to delayed c-Jun expression), it is clear that the failure of aged SCs to efficiently acquire a repair phenotype after nerve injury would impinge on axonal regeneration. In the future, it will be of great interest to determine both the exact cause for these alterations in the SC compartment and whether they contribute to other age-associated disorder of the PNS, such as peripheral neuropathy.

It is interesting to note that SCs are potentially unique among adult, differentiated cells in that, after injury, they undergo a phenotypic reprogramming process that resembles trans-differentiation (Arthur-Farraj et al., 2012) and acquire a de-differentiated highly proliferative almost stem cell like state. Given the well-known alterations that occur in stem-cell compartments during normal aging, it is perhaps not surprising that SCs, which undergo major transcriptional and phenotypic changes after injury, are relatively vulnerable to age-acquired

alterations. Similarly, glial cells in the central nervous system also exhibit an age-associated decline in function. For example, the differentiation potential of oligodendrocyte progenitor cells declines with age, resulting in decreased myelination (Shen et al., 2008). Unlike what we observed with SCs, however, this diminished function in CNS glia results in part from extrinsic signaling via hematopoietic cells (Ruckh et al., 2012).

Finally, our data may have bearing on therapeutic strategies for promoting neural repair in aged patients, where replication of a youthful extrinsic environment should be conducive to greater recovery. Although rare, limb transplants are now successfully performed and our work would suggest that the age of the donor may influence outcomes.

ACKNOWLEDGEMENTS

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EXPERIMENTAL PROCEDURES

Methods

Mice.

Young (8-12 week old) and aged (24-26 month old) C57/BL6 mice were obtained from Charles River and the National Institute of Aging (NIA), respectively. Thy1 YFP+ animals were bred in house, originally obtained from Jackson Labs, line # 003709. Animals were housed and handled in accordance with protocols approved by the Administrative Panel on Laboratory Animal Care (APLAC) of Stanford University or in full accordance with the IACUC guidelines of Children's Hospital Boston.

Sciatic nerve crush.

All surgical experiments were performed under 2.5% isoflurane on either young adult mice (8-12 weeks old) or aged mice (24-26 months old). Sciatic nerve crush injury was performed as previously described (Ma et al., 2011). Briefly, the sciatic nerve was exposed at mid-thigh level on the left side of the animal and crushed with smooth forceps for 30 seconds. For experiments involving Schwann cells (Fig 3): Mice were anesthetized using isoflurane. Upper thigh was shaved and sterilized using isopropanol. A 1 cm incision was made using a scalpel and nerve was visualized via blunt dissection using forceps. The left sciatic nerve was crushed at mid-thigh for 5 seconds using forceps marked with sterile graphite to

mark the crush site. Carprofen (5mg/kg subcutaneous) was administered for analgesia.

Sciatic nerve graft.

For Thy1 experiments, 1 cm long segments of the sciatic nerve (from mid-thigh level to the site of trifurcation) from either young or aged animals were acutely harvested and grafted using 10-0 nylon sutures onto the sciatic nerve just below the sciatic notch of 8 week old Thy1 YFP mice. These nerves were only sutured at the proximal sight and the distal side was left as a free ending. For nerve graft studies involving behavioral assessments, 3 mm of the sciatic nerve was removed from the host mice and was replaced with a 1cm long sciatic nerve graft of either young or aged animals. The proximal and distal ends were sutured with 10-0 nylon.

Assessment of sensory recovery using a pinprick assay.

Pinprick assay was performed as described previously (Ma et al., 2011). Briefly, mice were habituated on wire mesh cages for 30 minutes, until calm, and then tested for pain responses on the hind paw using a small insect needle. All experiments were repeated by at least 2 separate investigators. In the case of nerve graft studies, experiments were conducted blinded to the age of the donor graft. The person who performed the surgeries never participated in the behavioral experiments.

Assessment of motor recovery by toe-spreading test.

Toe-spreading assay was performed as described previously (Ma et al., 2011). Briefly, mice were gently covered with a piece of cloth and lifted by the tail, uncovering the hind paws for clear observation and the number of toes and extend of toe-spreading was measured for the injured side and compared to the uninjured paw. Again, at least two separate investigators made independent assessments for each experiment. Nerve graft studies were completed blind to age of donor graft and the surgeon did not participate in behavioral studies.

Microarray analysis.

Common pools of RNA were derived from ipsi- (injured) and contralateral (uninjured) L4 and L5 DRGs or ipsi- and contralateral sciatic nerves (1cm long segments distal to the site of injury), amplified, and hybridized to Illumina beadchip arrays. All microarray experiments were conducted in biological triplicates, except for the DRGs from old naive animals, which were done in duplicate. Each replicate was composed of tissue from at least 2 separate animals. All analyses were carried out either in Excel or in GenePattern (www.genepattern.org) using the Multiplot module.

Quantification of neurite outgrowth.

DRG sensory neurons were purified as described previously (Costigan et al., 1998). 1,500-2,000 cells were plated on laminin and fixed 17 hours after plating

(for naive) or 12 hours after plating (for pre-conditioned). Quantification was completed using Neuromath as described previously (Ma et al., 2011). Quantification was completed blind to age or condition and repeated three separate times.

Parabiosis.

Parabiotic pairs were joined as previously described (Ruckh et al., 2012). All animals used for parabiotic pairings were male, from congenic strains to avoid immune rejection between the partners. Parabiotic pairings included isochronic-young pairs (two young mice joined, each 8 weeks old), isochronic-old pairs (two old mice joined, each 24 months old -- all died) and heterochronic pairs (a young mouse joined to an old one, respective ages same as above).

Statistics.

All statistics were completed in GraphPad Prism version 6 for Macintosh.

Western Immunoblotting

Sciatic nerves were dissected from young and old mice at indicated days after sciatic nerve injury. Protein lysates from sciatic nerve were extracted in the presence of a protease cocktail tablet (Roche Diagnostics) using RIPA extraction buffer by homogenizer and cell debris was removed by centrifugation (4°C, 15min). Protein concentrations were determined by using BCA protein assay kit (Pierce). Equivalent amounts of protein were loaded and separated by SDS-

PAGE and subsequently transfer to an Immobilon-P PVDF transfer membrane (Millipore). After washing in TBS containing Tween-20, blots were blocked in 5% milk for 1h at room temperature and incubated with rabbit polyclonal antibodies against p-ERK (Cell Signaling, 1:500), total ERK (Sigma, 1:1000), p-JNK (Cell Signaling, 1:500), total JNK (Cell Signaling, 1:500), ATF3 (Santa Cruz, 1:500), total c-Jun (Cell Signaling, 1:500), and a mouse monoclonal antibody against GAPDH (Chemicon, 1:2000). HRP-conjugated secondary antibody (anti-rabbit, anti-mouse, Pierce, 1:5000), an ECL kit (Pierce) and autoradiography film (Genesee Scientific) were used following the manufacturer's protocol for signal detection.

Immunohistochemistry:

Sciatic nerves were harvested distal to crush by perfusing mice with ice cold PBS followed by cold 4% paraformaldehyde. Nerves were postfixed for 5 hours at 4°C. For myelin staining, epineurium was carefully removed and axon bundles were separated along natural divisions using forceps. 3-4 small (~3mm) segments of these bundles were cut at approximately the location of the nerve trifurcation. Segments were permeabilized in ice-cold methanol for 20 minutes and rinsed 3x10 minutes in blocking buffer (10% normal donkey serum,1%Triton X-100 in PBS) at room temperature (RT). Nerve bundle staining: Briefly, segments were blocked in blocking buffer overnight at 4°C. Segments were incubated in primary antibodies: goat anti p75 (Neuromics,1:500), rabbit anti-MBP (DAKO 1:100), diluted in blocking buffer at 4°C for 72 hours with rocking

followed by 3x10 minute washes at RT and 6x1 hour washes in blocking buffer at 4°C. Secondary antibodies (donkey anti-goat 488, donkey anti-rabbit 563, donkey anti-rat 633, Invitrogen) were diluted 1:1000 in blocking buffer. Segments were incubated in secondary antibody for 48 hours at 4°C with rocking and rinsed as after primary antibody incubation followed by 3 clearing steps in 25%, 50%, and 75% glycerol, respectively, for 24 hours each at 4°C. Nerve bundles were mounted in glycerol-based Vectashield mounting media with DAPI.

For staining of sciatic nerve cross-sections, post-fixed nerves were cryoprotected by sinking in 30% sucrose overnight. Frozen nerves were crysosectioned into 10-μm sections. Sections were permeabilized with ice-cold methanol for 10 minutes and blocked in blocking buffer (10% donkey serum, 0.2% Triton X-100) for 1 hour at RT followed by overnight incubation in primary antibody: goat anti p75 (Neuromics, 1:500), rabbit anti S100 (DAKO, 1:500), rat anti CD68 (AbD serotec 1:1000) at 4°C. Following 3x10 minute PBS rinses at room temperature, slides were incubated overnight in secondary antibodies (Invitrogen, 1:1000) at 4°C, rinsed 3x10 minutes in PBS and mounted with Vectashield plus DAPI.

Quantification

Quantification was performed using National Institutes of Health Image J. Images were acquired using Zeiss Axio Imager M1 and Axiovision software. Images were analyzed blind.

Internalized myelin quantification: Image of nerve bundle was cropped to uniform size and MBP signal threshold was adjusted to reveal areas of intense MBP accumulation. Area occupied by MBP/area of nerve in cropped image was determined.

P75/S100 quantification: For each cross-section, S100 and p75 signal threshold was adjusted to exclude background signal. A region of interest (ROI) was created based on the S100 positive area of the cross section. This ROI was applied to the p75 image and the percent of the ROI pixels positive for p75 was determined.

Thy1 YFP+ fibers quantification: Images of nerve sections from each distance from the suture site were taken at 10x magnification (at least 3 separate nerve images per animal per distance). The images were imported into ImageJ, converted to black and white, converted into a binary image, and the number of "masks" was counted for each image. This was done in exactly the same procedure for each image, and all images were taken with the same exposure time.

CHAPTER THREE

EXPANDED DISCUSSION, ALERNATE APPROACHES AND CONCLUSIONS

Neuronal intrinsic growth

In the context of regeneration in general, it is intriguing to observe that DRG sensory neurons appear relatively unaffected by age, both functionally in terms of neuronal intrinsic growth, as well as transcriptionally, where very few alterations were seen by microarray profiling **(Figure 2.3)**. While not included in the main manuscript due to the low number of animals, the slow-regeneration phenotype of aged animals did not appear to be rescued even by enhancing neuronal intrinsic growth *in vivo* **(see Appendix)**. This was accomplished by testing the functional recovery after sciatic nerve crush injury in 2-year old hsp-27 over-expressing mice. Hsp-27 has been demonstrated to enhance axonal regeneration and Western blot analysis showed continued expression of the construct at 2-years of age (data not show) (Ma et al., 2011).

Although our data strongly suggest that a failure in neuronal intrinsic growth in aged animals is likely not the primary problem, it is always difficult to show something is not the case, and it remains possible that we have overlooked some subtle neuronal defect. For example, one caveat of our functional assays is that we essentially took "snap-shots" of neuronal growth on laminin, a permissive substrate. To get a sense of temporal kinetics, it would have been worthwhile to complete time-lapse imaging of aged and young neurons in culture, as well as to look for defects on other substrates, such as on the non-permissive myelin. Similarly, had we performed microarray profiling of aged and young DRGs at

various time-points of injury, it is possible we would have detected a defect early in the course of injury. Still, the broad picture of neuronal responses with age is that they remain intact.

As discussed earlier in the thesis, there is ongoing debate in the field of axonal regeneration as to whether failure of axonal regeneration, either the CNS or the PNS, is truly influenced by a non-permissive environment. Although this thesis work does not speak to regeneration in the CNS, it does highlight the fact that a non-permissive environment can hinder regeneration even when intrinsic growth is robust or increased. Thus, while generally speaking overcoming inhibitory signals and enhancing intrinsic growth have been seen as two distinct approaches, it would be worthwhile to synergize these strategies. For example, one could imagine a triple deletion of PTEN, SOCS3 and PTPsigma in retinal ganglion cells to promote regeneration in the CNS, or simultaneously enhancing both ATF-3 and Schwann cell responses in the PNS.

Our finding of unaltered neuronal properties with advancing age is also interesting in the more general context of normal aging. Given that other long-lived post-mitotic cells are particularly susceptible to the aging process from factors such as oxidative stress, DNA damage and mitochondrial dysfunction (Geiger et al., 2013) our results imply that DRG neurons may have specific mechanisms for countering or preventing age-accumulated deleterious effects. Do DRG neurons have specialized or more active DNA repair pathways? Are

stress responses or protein degradation pathways more active in these cells? In collaboration with Amy Wagers, we attempted to assess the DNA-damage state of 2-year old sensory neurons and Schwann cells, however, these experiments failed along technical lines (purifying the cells ubiquitously caused DNA damage). Regardless, understanding what allows peripheral neurons to maintain themselves through advancing age may lead to greater insight as to how to maintain somatic cells in general.

These issues are particularly important in the context of neurodegenerative disease. Thus far, it is extremely unclear how changes to neurons during normal aging might alter the disease progression of neurodegenerative disorders, or whether there are substantial changes at all. One natural extension of this work would be to understand how neurons age in the CNS. Although a hallmark of many neurodegenerative diseases is the aggregation of protein(s) within cells, it is often unknown whether these proteins are actually involved with disease progression or are simply acting as a marker. While many neurodegenerative diseases result in the death of neurons, it is perhaps incorrectly assumed that the majority of changes result from the neurons themselves. Thus, it would be interesting to complete neuron-specific expression profiling and/or proteomics to determine whether and how neurons age in the CNS, and whether they do so differently in the background of disease.

The role of macrophages

In this thesis work, we also addressed the role of circulating and immune factors, including macrophages, in hindering axonal regeneration in aged animals. Although we used a parabiosis paradigm to test the role of circulating macrophages, there are other strategies we could have employed, such as performing age mis-matched bone marrow chimeras or even bone marrow transplants. We chose parabiosis because it is considered the least disruptive method for accomplishing chimerism of blood cells; however, one benefit of these alternative approaches is that they would have provided a nearly compete replacement of donor cells, perhaps better addressing the question. In particular, the possibility that aged macrophages exert a "negative" effect on regeneration is difficult to exclude in a chimeric model, as perhaps even a few aged macrophages are enough to impair axonal regeneration.

In a tangential sense, these data also add fuel to the fire about the often debated role of macrophages in PNS regeneration. As mentioned earlier, some of the strongest evidence in support of key macrophage involvement in promoting PNS regeneration has come from studies in which myeloid cells were depleted and subsequent axonal regeneration was shown to be impaired, such as in Barrette et al. (Barrette et al., 2008). In that study, the authors demonstrated that myelin clearance was delayed in the sciatic nerves of animals lacking innate immune

cells, and suggested, as many others have, that the critical role for macrophages is in clearing inhibitory myelin from the site of injury.

While not directly addressed in our manuscript, our data is in conflict with these conclusions, and further poses questions about the true function of macrophages in peripheral regeneration. As seen in **Figure 2.6** in Chapter 2, myelin is undoubtedly being sequestered and engulfed by Schwann cells until at least one week after injury (the longest observed time-point). In fact, from our histological work, it appears that there is very little, if any, exogenous myelin outside of Schwann cells during these time points. Thus, if macrophages are indeed the primary myelin debris clearers after nerve injury it is extremely unclear how or why they are obtaining myelin. More puzzling, after 7 days, when macrophages are at their peak numbers, axons in the PNS are well on their way to regenerating, as by day 10 or 11 they have formed functional connections with their downstream targets in the skin **(Figure 2.1)**, thus macrophage-dependent debris clearance does not appear to affect axonal regeneration at early time-points, even though their absence is supposedly inhibitory. Do macrophages perhaps clear dying or dead Schwann cells themselves? Is there a deeper Schwann cell/macrophage interaction, either through cell-cell contact or via secreted factors? Clearly more careful work will need to be done to fully understand the role of macrophages in promoting regeneration, starting with detailed observations of Schwann cell and macrophage interactions. Molecular interrogation of macrophages within the DRG and distal stump at various time-

points after injury in the nerve might also serve to help elucidate whether there are kinetic changes to macrophage phenotypes or if they secrete particular factors capable of impinging on regeneration.

Finally, these results also underscore the differences in macrophage function with respect to the CNS and PNS. As mentioned earlier, the differentiation and myelinating potential of oligodendrocyte progenitor cells (OPCs) declines with advancing age (Ruckh et al., 2012). Unlike in the PNS, however, results from those studies indicate critical roles for macrophages in stimulating OPC differentiation. Furthermore, using parabiosis, the same study implies that there are macrophage-intrinsic defects with aging, and that these defect bear directly on glial cell function in the CNS.

The role of Schwann cells

While our work raises a number of issues related to macrophage function and contribution to regeneration, it also strengthens growing notions of Schwann cell importance. Although our data overwhelmingly provides a model in which age-associated Schwann cell dysfunction leads to poor axonal regeneration, it is nonetheless difficult to completely rule out other mechanisms, such as differences in fibroblasts, resident macrophages or extracellular matrix cues, however unlikely. To this end, future experiments could potentially directly address the role of Schwann cells through age-mismatched Schwann cell

transplants. In these cases, however, it is unclear whether Schwann cells injected into the sciatic nerve *in vivo* would have the capacity to engraft or compete against the resident Schwann cells already occupying the niche. A second experiment that we attempted, which failed for technical reasons, was to infect aged Schwann cells *in vivo* with an adenovirus loaded with a c-Jun over-expressing construct. This would have been another strategy to directly test our suggested molecular mechanism.

How exactly do Schwann cell defects in de-differentiation lead to impaired axonal regeneration? The answer is likely to be contained not within one Schwann cell function, but many. As demonstrated in Chapter Two, there is some evidence to suggest that Schwann cell phagocytosis is impaired as a result of poor de-differentiation. This evidence is supported by recently generated data showing that aged Schwann cell phagocytosis of myelin *in vitro* is also impaired (data not shown). Thus, impaired Schwann cell clearance of myelin may be one major factor.

In addition, it is possible that reduced secretion of growth factors or reduction in the expression of inhibitory molecules also plays a role in reducing axonal regeneration in aged animals. In support of this view, our microarray data shows fewer growth factor-associated transcripts after injury in old animals **(Figure 2.6)**. Although preliminary, we have also generated some data implying that Schwann cells can directly modify the growth potential of neurons *in vitro* and that the age

of both cell types may affect this growth **(see Appendix)**. It will be interesting in the future to test the growth promoting effects of both young and aged Schwann cell conditioned media on neuronal cultures as well as to identify potential molecules involved. At least one previously uncharacterized EGF family member growth factor, betacellulin, comes up in our array data of the aging distal stump **(Figure 2.6)**.

A recent study suggested that Schwann cells themselves may actually form physical barriers to axonal regeneration (Kang and Lichtman, 2013). One possible explanation of these observations is that Schwann cells in their myelinating state do not adequately form so-called bands of Bungner, essentially failing to laydown the "roads" on which newly regenerating axons can grow. Thus, while difficult to directly test, a failure of aged Schwann cells to de-differentiate may result in physical impediments.

Finally, as is the case with the neurons, a fundamental question that this thesis work poses is whether the age-associated aberrations we observe in Schwann cells are unique to these glia, or whether cells of the glia lineage are generally susceptible to age-induced dysfunction. Again, this is important in the context of neurodegenerative disorders, where it is not known how an aging glial environment might influence the course of disease progression. One particularly intriguing study demonstrated that wild-type neurons will die when cultured with glia that lack SOD1, or with mutant human SOD1, the gene responsible for many familial cases of amyotrophic lateral sclerosis (Di Giorgio, 2007). As superoxide dismutase (SOD) plays a key role in regulating oxidative stress, it is not farfetched to think that glia acquire dysfunction with normal aging.

Concluding remarks

Overall, I hope that you have found this thesis as enjoyable to read as it has been to produce the work contained within it. I feel this thesis work has taken an important step forward in understanding a basic biological question -- why does aging impair neuronal regeneration? -- while simultaneously using an aging model to examine fundamental questions about how peripheral nerve regeneration works.

APPENDIX

Forward

Found here are complete rationale, descriptions and methods that are mentioned and tangentially relevant for the thesis, but not part of the main body of work **(Chapter 2)**. They are useful additions to the introductory and discussion chapters.

Global view of regeneration-associated genes

Although various markers associated with axonal regeneration have been identified, I wished to explore the transcriptional profile of injured vs. naive DRG neurons from a genome-wide point of view. To accomplish this, I utilized previously generated data (Seijffers et al., 2007) and plotted the expression of each gene in injured DRG neurons against its corresponding expression in naive DRG neurons. These results visually demonstrate that several hundred genes are activated in the DRG upon nerve injury in a predominantly up-regulated fashion **(Figure A1)**. Although we tend to think of regeneration-associated genes as being limited to a handful of transcription factors (ATF-3, c-Jun, Sox11) along with a couple of other makers (GAP-43, SPRR1A), this analysis shows that the injured-induced transcriptional program is widespread, being comparable to two completely different cell-types of the same lineage. Furthermore, this analysis shows the need to study regeneration-associated transcriptional programs from a "systems level" point of view, incorporating each gene and the potential modules it regulates into our mode of thinking.

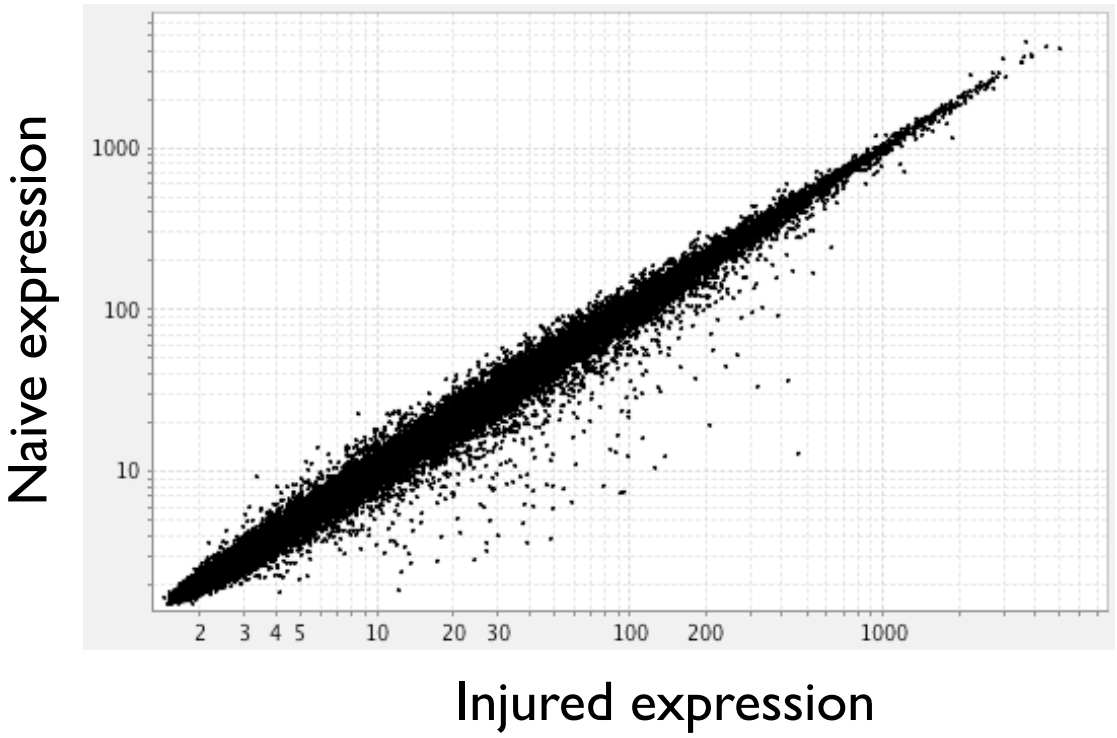


Figure A1: Injury-induced transcriptional response in DRG neurons. Expression vs. expression of whole DRG transcriptomes under either steady-state (naive) or injured (5-days after sciatic nerve crush injury) conditions. Microarray data used from Rhona Seijffers (Seijffers et al., 2007). Expression represents averages from n=3 biological replicates for both naive and injured DRGs.

Assessing the role of neutrophils in peripheral regeneration

Recent work has revealed novel ways in which immune cells, in particular cells of the innate immune system, participate in axonal regeneration. In the central nervous system, it has been found that neutrophils can promote optic nerve regeneration through the secretion of a protein called oncomodulin (Kurimoto et al., 2013). Neutrophils are among the first responders during an inflammatory response, and given their large numbers early during the course of nerve injury in the PNS, I wondered whether neutrophils may exert an influence on axonal regeneration in the periphery as well.

To test this hypothesis, I depleted neutrophils in cohorts of mice *in vivo* using a highly specific antibody, Ly6G. Control cohorts received a corresponding isotype control (rat IgG2A). After neutrophil depletion was established, sciatic nerve crush injury was performed and restoration of sensory function over time was monitored through pinprick assay. As seen in **Figure A2**, no difference in functional recovery was seen between mice lacking neutrophils and mice receiving control antibody.

Given results in the CNS showing a critical role for neutrophils (Kurimoto et al., 2013), these results were surprising, but emphasize the differences between these two systems. Perhaps there is a quantitative or qualitative difference in the neutrophils in the CNS vs. the PNS. For example, it is possible that PNS neutrophils do not secrete oncomodulin, or that regeneration is so robust in the

PNS that any contribution from neutrophils is not necessary. Another possibility is that some other cell-type or signal acts to stimulate oncomodulin production. In any case, we found that neutrophils are dispensable for functional recovery of the PNS.

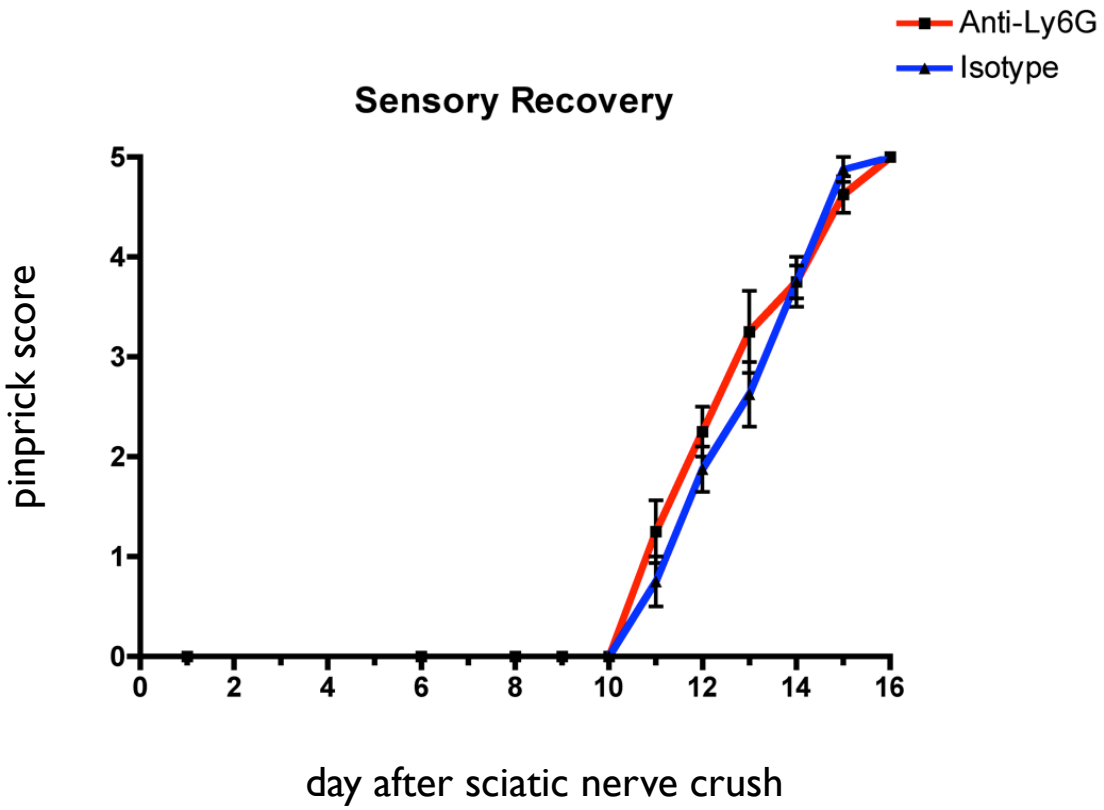


FIGURE A2: Neutrophil depletion does not alter functional recovery after nerve injury. Restoration of sensory recovery was measured in cohorts of C57 mice with or without depletion of neutrophils. Mean +/- S.E.M is plotted; n = 8 mice for each group.

The role of non-neuronal cells in the pre-conditioning effect

As gleaned from the terminology, neuronal intrinsic growth has consistently been viewed as a neuronal-centric process; that is to say, one that is initiated and maintained by the neuron itself. Despite this, there is substantial evidence to suggest that this is not the complete picture. For example, as mentioned above, inducing an inflammatory response in the optic nerve can activate intrinsic growth in retinal ganglion cells. In the PNS, too, there are hints that robust neuronal intrinsic growth may in part dependent upon non-neuronal factors. For instance, culture of dissociated DRG sensory neurons will grow neurites *in vitro*, but the extent and rate of growth will never compare to that found in pre-conditioned neurons. This observation strongly suggests that "time" is not the only factor in activating regeneration-associated genes, but rather some *in vivo* component other than the neuron is participating.

Given this, we wondered whether the presence or absence of non-neuronal cells could influence the pre-conditioning effect *in vitro*. To accomplish this, we either purified DRG neurons extensively, through multiple gradients (BSA and Percoll), or intentionally retained most of the non-neuronal cells by not purifying them at all. We then plated these cells on a permissive substrate for 5 days, re-plated them, and assessed neurite outgrowth. As seen in **Figure A3**, the presence of non-neuronal cells greatly enhances the *in vitro* pre-conditioning response. Unfortunately, substantial neurite outgrowth beyond a certain threshold makes quantification using automated software impossible.

Though preliminary in nature, and should be replicated more, these results add intriguing evidence to the case that the pre-conditioning effect may be in part driven by non-neuronal signals. The identity of these cell-types and signals remain completely unexplored, however, and future work should address this issue.

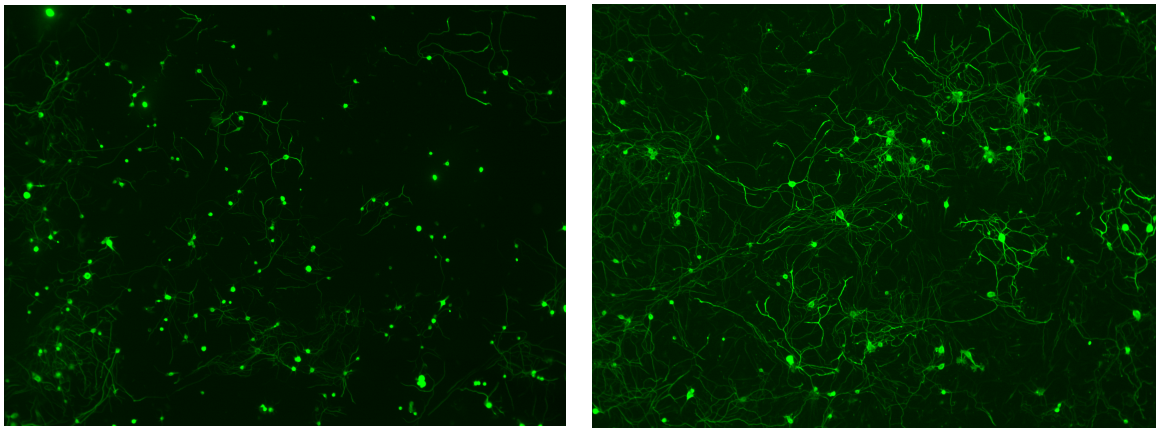


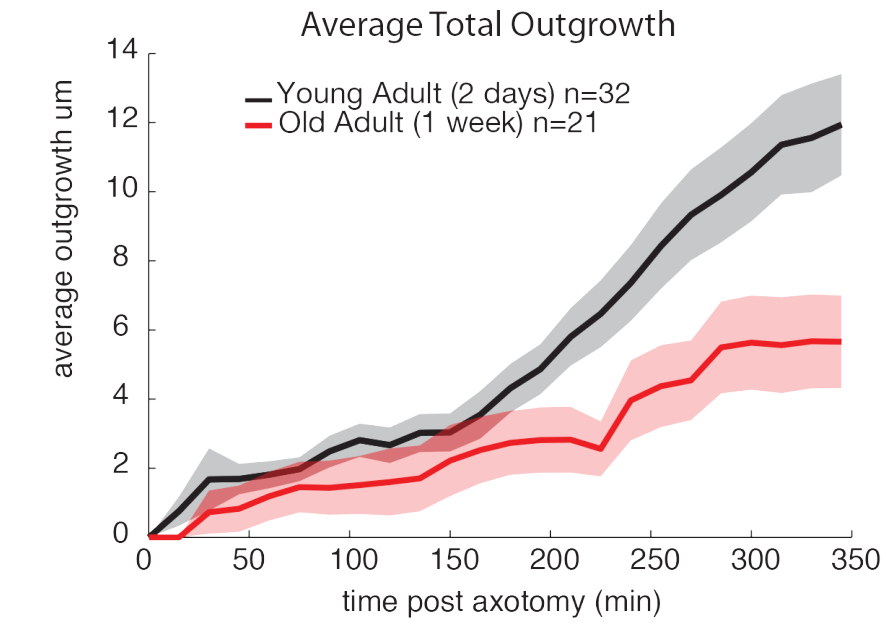
FIGURE A3: in vitro pre-conditioning is enhanced by the presence of non-neuronal cells. Representative images of DRG neurons cultured in the presence (right) or absence (left) of non-neuronal cells within the DRG. ~1000 neuron were plated per well.

Assessing age-associated axonal regeneration in C. *elegans*

At the outset of this thesis work several years ago, an age-associated decline in axonal regeneration had been observed histologically in a number of murine models, but whether this process was conserved in lower organisms had not been addressed. Axonal regeneration readily occurs in sensory systems of C. *elegans*, and as such, we wished to know whether aging impairs regeneration in a conserved manner. In collaboration with Christopher Gabel's group at Boston University, we performed specific laser ablation of individual sensory axons in cohorts of young (2 days) or old (1 week) C. *elegans* and tracked the regrowth of these axons over time using time-lapse video imaging. We observed a very robust effect of aging on both the time required to initiate axonal outgrowth as well as the total outgrowth over time **(Figure A4)**, mirroring data we generated in aging mice **(see Chapter 2)**.

Because Wallerian degeneration does not occur in C. *elegans*, and in fact, axons simply regenerate around the cut axon, these data argue for a neuronal-intrinsic defect in C. *elegans*. Indeed, a recent publication confirmed that intrinsic mechanisms regulate the developmental decline in neuronal regeneration in worms (Zou et al., 2013). Thus, we conclude that age-related defects in C. *elegans* are likely the result of different mechanisms than in a mammalian system.

FIGURE A4: Axon regeneration is slower in aged C. *elegans*. Individual neurons of young (2-day) and aged (1-week) worms were ablated with lasers and subsequent axonal regeneration was measured using time-lapse photography. **A)** Total neurite outgrowth. **B)** Average time between laser ablation and initiation of neurite outgrowth. Plotted is mean +/- S.D.; n = 20-30.



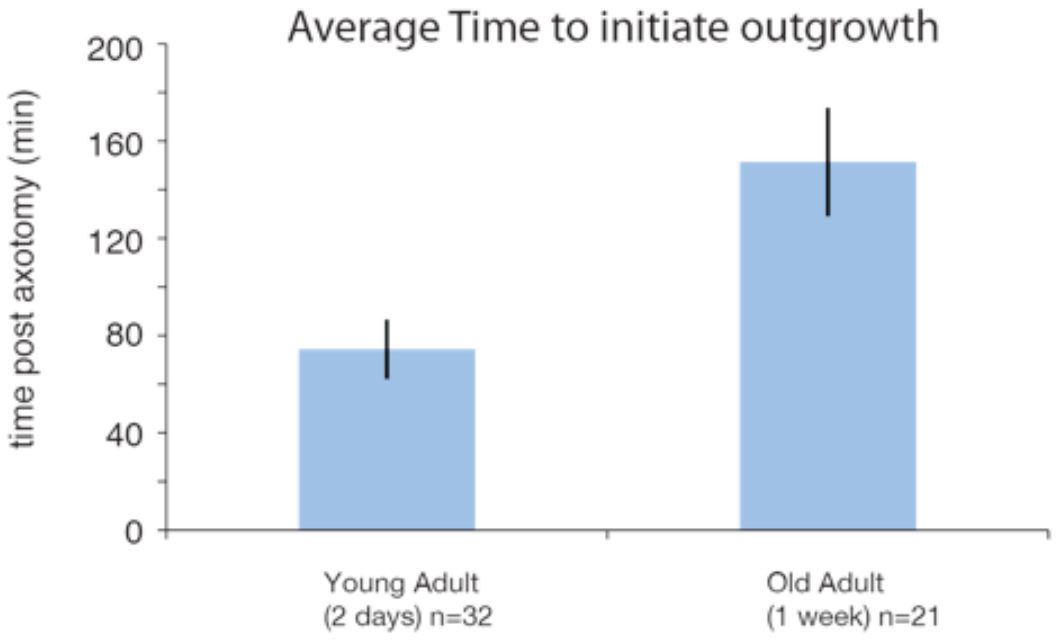


FIGURE A4 (continued): Axon regeneration is slower in aged C. *elegans*.

Comparing transcriptional injury responses in the DRG and the distal stump

In thinking about what defines regeneration-associated genes within DRG neurons, I have often noted the unusual overlap between genes known to play important roles in neuronal intrinsic growth, and those known to be important in regulating Schwann cell injury responses. For example, c-Jun is both a critical transcription factor in promoting axonal regeneration in neurons and a master regulator of Schwann cell de-differentiation. Among others, GAP-43 and ATF-3 are also highly up-regulated in the distal stump after injury **(see Chapter 2)**. Given this, I wondered more generally just how much of the transcriptional response is shared between the DRG and distal stump after nerve injury.

To address this question, I first combined microarray data generated from 4 days after nerve injury from both the DRG and distal stump in young animals. I then compared the expression patterns before and after injury of each of these tissues on a Fold Change vs. Fold Change plot to get a rough estimate of how many genes activated after injury in one tissue were also activated in the other. Using 2-fold change after injury as a standard cut-off, this analysis revealed that roughly 60% of the genes up or down regulated after nerve injury in the DRG are also activated in the same way in the distal stump **(Figure A5)**. As seen along the X and Y axis, there are also a number of tissue-specific genes, as well as genes that exhibit the opposite mode of activation in one tissue versus the other.

Still, as shown by the large number of transcripts falling along the Y=X line, however, the expression of a surprising number of genes appear to be coordinated in both tissues.

There are at least two important points to be made from this analysis. The first is a cautionary tale when interpreting data from whole mouse knockout studies, or similar strategies that wish to address either Schwann cell responses or neuronal intrinsic growth without methods to confine the experiment to a single compartment. This is because it is a very likely that altering the function of a gene in both the neuronal and Schwann cell compartments would yield un-interpretable data. The second point is a question: given this degree of transcriptional overlap, a degree that seems unlikely to be coincidental, what is the purpose of sharing injury programs? Certainly it is a question that begs closer examination in the future and suggests intriguing possibilities regarding axon and Schwann cell communication.

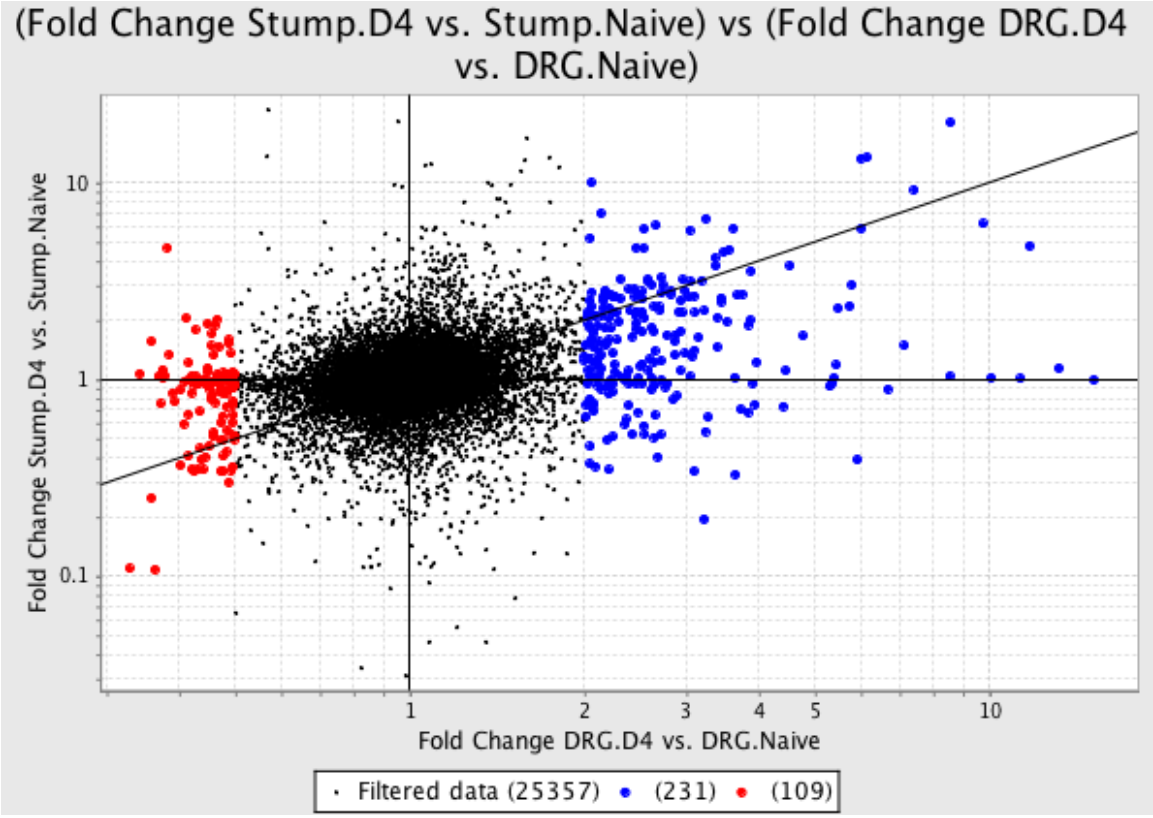


FIGURE A5: Analysis of injury-induced transcripts reveals broad similarities between programs utilized in the DRG and distal nerve stump. Fold-change vs. fold-change plot of expression data generated from naive and injured (4 days post nerve crush) DRG and distal nerve stump. Transcripts highlighted in blue represent fold change greater than 2 after injury in the DRG while those in red represent fold change less than 0. Dots are representative of averages of 3 independent biological replicates.

Enhancing neuronal intrinsic growth in aged animals *in vivo*

In **Chapter 2**, we concluded that neuronal intrinsic growth was unlikely to be a major factor influencing the aging regeneration phenotype. In addition to the experiments described in **Chapter 2**, we also performed a preliminary experiment intended to push the question of intrinsic growth to a more extreme position. In this case, we not only wished to know whether intrinsic growth was failing in aged animals, but whether boosting intrinsic growth could rescue the aging phenotype regardless of its source. To accomplish this, we took advantage of Hsp27 over-expressing mice, which show accelerated axonal growth after nerve injury (Ma et al., 2011). We performed sciatic nerve crush injury on a small cohort of ~24 month old Hsp27 transgenic mice and compared the rate of restoration of sensory functional recovery over time to previously generated data from young and aged animals. Interestingly, we found that functional recovery of aged Hsp27 animals compared extremely well to that of non-transgenic aged animals **(Figure A6)**. Although these data were not included in the manuscript because of low n number and lack of adequate control animals, they suggest that boosting neuronal intrinsic growth cannot accelerate regeneration in aged animals. These results are consistent with a dominant extrinsic effect of aging on regeneration as proposed in **Chapter 2**.

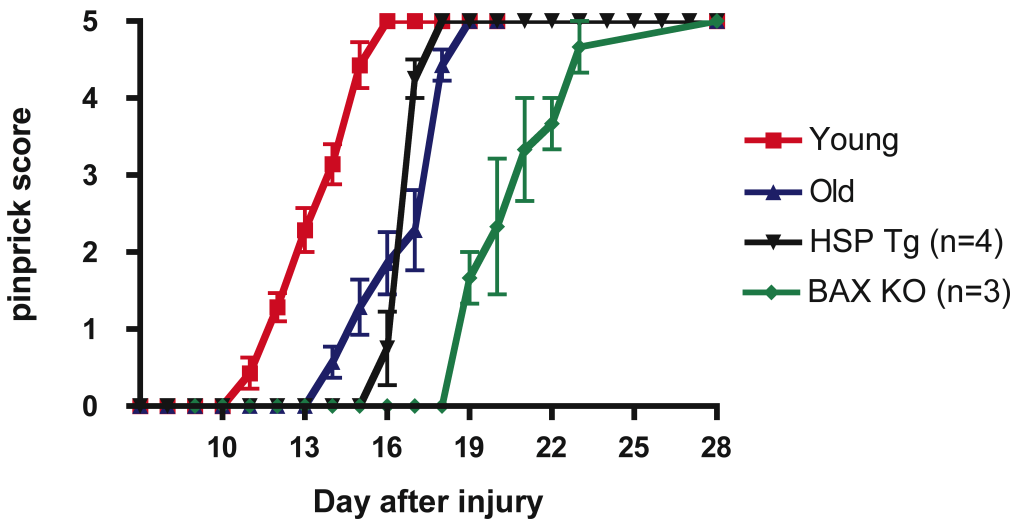


FIGURE A6: Enhancing intrinsic growth does not rescue the aging phenotype. Rate of restoration of sensory function over time in 2 year old Hsp-27 transgenic mice. Plotted is mean +/- S.E.M., n=4. Red and Blue represent previously generated data **(see Chapter 2)** for sake of comparison.

Assessing age-dependent effects of Schwann cells in promoting neurite outgrowth *in vitro*

Among the possible ways that aged Schwann cells might influence the course of axonal regeneration is through reduced secretion of growth-promoting factors or by functionally providing a less adequate substrate for axonal growth. To test whether aging Schwann cells might directly hinder neurite outgrowth, or whether young Schwann cells might enhance growth, we co-cultured young and aged Schwann cells with young and aged neurons and assessed neurite outgrowth after 17 hours on a permissive substrate. While there appears to be an interesting trend, overall the effect of Schwann cells on growth did not have a statistically significant effect **(Figure A7)**. We did not try Schwann cell conditioned media, but this would have been worthwhile.

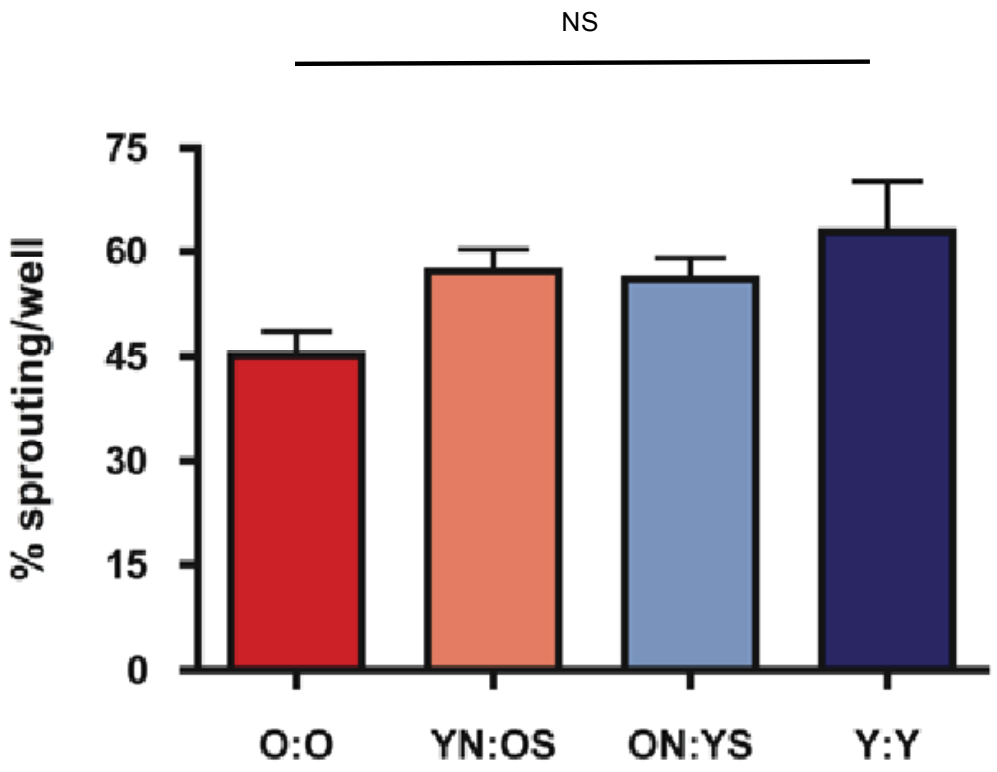


FIGURE A7: Schwann cell and neuronal co-culture experiments.

Old or young Schwann cells were cultured with old or young DRG neurons and subsequent neurite outgrowth was measured after 17 hours. O=old Y=young; YN:OS = young neurons, old Schwann cells, ON:YS= old neurons, young Schwann cells. Plotted is mean +/- S.E.M, n = 4 wells per condition. NS = not significant by one-way ANOVA.

Methods

Microarray profiling and analysis.

Microarray profiling was generally performed according to methods in **Chapter 2**. In brief, commons pools of RNA were derived from L5 and L6 DRGs from cohorts of C57BL6/J mice, amplified using standard Ambion kits, and the resulting cDNA hybridized to Affymetrix 430 whole genome mouse arrays. Analysis was conducted in Multiplot software, also described in **Chapter 2**.

Neutrophil depletion.

Neutrophils were depleted according to methods found in (Daley et al., 2008). Briefly, neutrophils were depleted by I.P. administration of 0.5mg Ly6G antibody (1A8 hybridoma from BioXcell) 3 days prior to sciatic nerve crush injury, and every 2 days after until full restoration of sensory recovery in the mice.

Schwann cell culture.

1 cm long segments of the sciatic nerve were harvested from C57 mice and allowed to incubate in DMEM for 2 weeks. This allows Schwann cells to proliferate and the nerve to degenerate. After incubation, nerves were placed in a mixture of collagenase and dispase at 37 degrees Celsius (as with DRGs) for 2 hours. Nerves were then directly titurated using a glass pasteur pipette in 2ml of DMEM. The resulting cell suspension was filtered and results in approximately 80-90% Schwann cells.

Mouse behavior.

Restoration of sensory recovery in mice was accomplished through the pinprick assay as described in methods in **Chapter 2**.

DRG and non-neuronal purification and culture.

Dissociated DRG neurons were obtained and cultured using methods described in **Chapter 2**. Non-neuronal cells were obtained by dissociating the entire DRG tissue as previously described, but without the use of neuronal cell purifying gradients (Percoll and BSA).

Femtosecond laser ablation and time-lapse imaging in *C. elegans*.

Laser ablation and imaging were performed according to previously described methods (Chung et al., 2013).

Transgenic mice.

Hsp27 transgenic mice were generated according to methods described previously (Ma et al., 2011).

SUPPLEMENTAL TABLES

IlluminaProbeID	Gene Symbol	Fold Change (Young injured v.s aged injured)
ILMN_2878542	Myl1	3.13
ILMN_2738825	Acta1	2.65
ILMN_2794342	Pmp2	2.45
ILMN_2997494	Lox	2.28
ILMN_1248316	Ptgds	2.24
ILMN_2666864	Atp2a1	2.19
ILMN_2717765	Ednrb	2.10
ILMN_2882658	Tnnc2	2.09
ILMN_2648669	Gpnmb	0.33
ILMN_1238886	Ccl8	0.33
ILMN_2712075	Lcn2	0.38
ILMN_2754364	Ltf	0.47
ILMN_2846865	Actb	0.49
ILMN_1228832	Ngp	0.50

Table S1: Differentially expressed DRG genes between young and aged after injury.

Red: genes over-expressed in young greater than 2-Fold. Blue: genes under-expressed in young greater than 2-Fold (less than 0.5).

IlluminaProbeID	GeneSymbol	Fold Change (young naive vs. aged naive)
ILMN_2875730	MUP1	5.79
ILMN_2737163	SQLE	4.76
ILMN_2688075	CYP51	4.55
ILMN_2600348	SQLE	4.48
ILMN_1225730	FDPS	4.31
ILMN_3065459	MUP2	4.17
ILMN_1239557	UGT8	3.12
ILMN_2614752	ELOVL6	3.09
ILMN_1229529	HSD17B7	3.08
ILMN_2443330	TTR	2.95
ILMN_2676379	PON1	2.90
ILMN_2823778	SC4MOL	2.84
ILMN_2654952	HMGCS1	2.80
ILMN_1247358	EMID2	2.80
ILMN_1253601	AACS	2.79
ILMN_1253806	COL1A2	2.65
ILMN_2543108	WDT3-PENDING	2.63
ILMN_1229547	SPON2	2.60
ILMN_1258629	COL3A1	2.60
ILMN_2794342	PMP2	2.59
ILMN_2594525	NSDHL	2.53
ILMN_2909105	2310047D13RIK	2.50
ILMN_2683958	COL3A1	2.45
ILMN_2607377	ITM2A	2.45
ILMN_2731446	SERPIND1	2.31
ILMN_1245272	INSIG1	2.28
ILMN_1216661	COL6A2	2.27
ILMN_1225835	MFAP5	2.27
ILMN_1215136	SCN3B	2.26
ILMN_2592953	SGNE1	2.26
ILMN_2768087	COL6A1	2.26
ILMN_1259388	COL6A1	2.25
ILMN_2734181	ACAS2	2.24
ILMN_2997494	LOX	2.23
ILMN_2594521	NSDHL	2.23
ILMN_2939303	PCYT2	2.16
ILMN_2687872	COL1A1	2.16
ILMN_1377923	ACTB	2.13
ILMN_2645275	MVD	2.11
ILMN_2814357	GM239	2.10
ILMN_1240445	STARD4	2.09
ILMN_2747031	DHCR24	2.07

ILMN_2874422	ANGPTL1	2.05
ILMN_2724226	4833439L19RIK	2.05
ILMN_2866856	H2-DMA	2.04
ILMN_2606693	INSIG1	2.03
ILMN_1246821	E130203B14RIK	2.02
ILMN_1250507	ELOVL4	2.01
ILMN_2604224	SEMA5A	1.96
ILMN_2766930	FAAH	1.96
ILMN_2877507	PIGQ	1.94
ILMN_2946970	THEM5	1.93
ILMN_2781458	TLCD1	1.92
ILMN_2794645	CYR61	1.92
ILMN_2762397	FBXO25	1.91
ILMN_2597485	PCYT2	1.89
ILMN_2619846	SLC25A1	1.88
ILMN_1245328	BB146404	1.88
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ILMN_2804166	IGSF9	1.85
ILMN_2604226	SEMA5A	1.85
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ILMN_1222111	COL15A1	1.84
ILMN_2894125	FOXI2	1.84
ILMN_2752940	DHCR24	1.83
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ILMN_1237021	MAG	1.74
ILMN_1255473	0610040B21RIK	1.74
ILMN_2657980	FAAH	1.74
ILMN_2729103	ADAMTS2	1.73
ILMN_1242246	SCAMP5	1.73
ILMN_1226356	AI427515	1.72
ILMN_1248837	TBXA2R	1.71
ILMN_1223598	SREBP2	1.71
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ILMN_2680262	POLR2G	1.71
ILMN_1232261	CATNAL1	1.70
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ILMN_1223594	LOC217066	1.70
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ILMN_2664884	TSPAN17	1.69
ILMN_2829636	DNAJB9	1.68
ILMN_2679386	GSN	1.68
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ILMN_2596499	BC058638	1.52
ILMN_2790373	SNN	1.52
ILMN_3059326	SPARC	1.52
ILMN_2607529	NR2F1	1.51
ILMN_1377919	TUBB2B	1.51
ILMN_1244977	H2-DMB1	1.51
ILMN_2433547	TRP53BP1	1.51
ILMN_1248714	DAF1	1.51
ILMN_2702266	CRYGS	1.51
ILMN_3154269	PRX	1.51
ILMN_2595597	ANLN	1.51
ILMN_1219915	DGAT2	1.51
ILMN_2893564	1190007F08RIK	1.51
ILMN_2883795	DCUN1D4	1.50
ILMN_2515784	TM4SF6	1.50
ILMN_1230411	KPNB1	1.50
ILMN_2594139	6330403K07RIK	1.50
ILMN_1223307	4933424M23RIK	1.50
ILMN_2596230	2410014A08RIK	1.50
ILMN_1248788	0610005K03RIK	1.50
ILMN_2916705	TUBA1A	1.50
ILMN_2610013	SHH	0.14
ILMN_1260428	MYL2	0.14
ILMN_2954987	MB	0.16
ILMN_2977331	MYLPF	0.16
ILMN_2882658	TNNC2	0.18
ILMN_2416670	TTN	0.19
ILMN_3128792	TTN	0.20
ILMN_2629581	COX6A2	0.20
ILMN_2608804	CKM	0.20
ILMN_2481133	TNNI2	0.21
ILMN_2666864	ATP2A1	0.21
ILMN_2933357	TCAP	0.21

ILMN_2789650	CSRP3	0.23
ILMN_1238886	CCL8	0.24
ILMN_2738825	ACTA1	0.25
ILMN_2648669	GPNMB	0.26
ILMN_2733708	RBP7	0.26
ILMN_1235070	NRAP	0.27
ILMN_2676012	MYBPC2	0.30
ILMN_2460136	TNNT1	0.30
ILMN_2691780	MYL3	0.31
ILMN_2503052	TNNC1	0.31
ILMN_2613038	1110033I14RIK	0.31
ILMN_2482209	TPM2	0.32
ILMN_2817864	CKMT2	0.32
ILMN_2747543	ACTN3	0.33
ILMN_2698052	CKMT2	0.33
ILMN_2797061	ACTN2	0.33
ILMN_2469018	TNNT3	0.33
ILMN_2757569	ENO3	0.34
ILMN_2649810	RPL3L	0.35
ILMN_2759365	ANGPTL4	0.36
ILMN_1216042	APOE	0.36
ILMN_2742068	CSRP3	0.37
ILMN_1227675	SLC4A1	0.38
ILMN_2992709	TREM2	0.38
ILMN_2749037	NDG2	0.38
ILMN_1218223	PVALB	0.38
ILMN_3064283	PDE4DIP	0.38
ILMN_2622671	ACSL1	0.41
ILMN_1222679	CIDEC	0.41
ILMN_2730425	RYR1	0.42
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ILMN_3008110	ACTN3	0.43
ILMN_1235372	HBB-B1	0.43
ILMN_2614655	GPNMB	0.43
ILMN_1229990	AGXT2L1	0.43
ILMN_2665133	TIMP4	0.44
ILMN_2629112	CRG-L1	0.44
ILMN_2805339	HSPB6	0.44
ILMN_2642417	MEST	0.45
ILMN_2918875	SLN	0.45
ILMN_2640097	AOX3	0.45
ILMN_2485323	TRF	0.45

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ILMN_2440530	VAT1	0.47
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ILMN_2708303	ACTN3	0.47
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ILMN_1245079	ADSSL1	0.49
ILMN_2765759	ASB2	0.49
ILMN_2958099	ADSSL1	0.49
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ILMN_1237871	LOC229665	0.50
ILMN_1241350	VSIG4	0.50
ILMN_2873444	FBXO32	0.50
ILMN_2741117	ANKRD23	0.50
ILMN_1234662	MB	0.50
ILMN_2774160	HSD11B1	0.50
ILMN_2488846	TMOD4	0.50
ILMN_2878542	MYL1	0.50
ILMN_3115917	HSD11B1	0.50
ILMN_1257299	G0S2	0.51
ILMN_1246800	SERPINA3N	0.51
ILMN_2727520	COX8B	0.51
ILMN_2599130	ALDOA	0.51
ILMN_2694179	CCL6	0.51
ILMN_1246139	CLDN11	0.52
ILMN_2473620	RSN	0.52
ILMN_2756046	GPR43	0.52
ILMN_2661366	BC018222	0.52
ILMN_2588249	S3-12	0.52
ILMN_1259206	HRC	0.52
ILMN_2873822	AEBP1	0.53
ILMN_2897617	RPS10	0.53
ILMN_2588815	PGAM2	0.53
ILMN_2864309	LOC100034251	0.53
ILMN_2617468	1810008K03RIK	0.53
ILMN_2733073	RYR1	0.54
ILMN_2665387	CORO1C	0.54
ILMN_2835683	H2-GS17	0.54
ILMN_2706269	HSPB1	0.54
ILMN_2760019	CXCL13	0.54
ILMN_2690122	SLC27A1	0.54
ILMN_2836982	DES	0.54

ILMN_2960114	CYP27A1	0.54
ILMN_2760979	TGFBR2	0.55
ILMN_2771219	1110001A05RIK	0.55
ILMN_2642681	ATF4	0.55
ILMN_2628892	PFKM	0.55
ILMN_1259322	PDK4	0.55
ILMN_1225204	2510004L01RIK	0.55
ILMN_2987339	MYBBP1A	0.55
ILMN_3136283	LDB3	0.55
ILMN_2661820	AGXT2L1	0.55
ILMN_3052430	CD63	0.55
ILMN_2815138	MYOM1	0.56
ILMN_3151722	NET1	0.56
ILMN_2715546	GPX3	0.56
ILMN_3049559	C4B	0.56
ILMN_2444217	TTID	0.56
ILMN_3154640	AFF1	0.56
ILMN_3137899	OTOP1	0.56
ILMN_2752994	FBXO32	0.56
ILMN_2968211	LGALS4	0.57
ILMN_2689880	GSTT2	0.57
ILMN_2688416	D11ERTD736E	0.57
ILMN_2619200	ERAF	0.57
ILMN_1250904	RTN2	0.57
ILMN_1225376	SMOC1	0.57
ILMN_1217969	GPIHBP1	0.57
ILMN_3153940	CMYA4	0.57
ILMN_2740151	CHPT1	0.57
ILMN_1216322	HMGCS2	0.57
ILMN_2604494	PODXL	0.57
ILMN_2712075	LCN2	0.57
ILMN_2692644	BC054059	0.57
ILMN_1214880	DMN	0.57
ILMN_2727503	IGFBP3	0.58
ILMN_2878071	LYZ	0.58
ILMN_3137570	PDLIM5	0.58
ILMN_2654896	RPL37A	0.58
ILMN_2944601	4933439C20RIK	0.58
ILMN_1244188	H6PD	0.58
ILMN_2863390	FCER1A	0.58
ILMN_2666990	EEF1A2	0.58
ILMN_3100812	GPX4	0.58
ILMN_2798129	C6	0.58

ILMN_2669714	PLTP	0.58
ILMN_1252076	LYZS	0.58
ILMN_2907370	OPTN	0.58
ILMN_3148662	SFRS5	0.59
ILMN_1240898	RPS21	0.59
ILMN_2671517	RPS15	0.59
ILMN_3126986	RPS24	0.59
ILMN_2955104	NACA	0.59
ILMN_1257631	APOBEC1	0.59
ILMN_1240746	MCPT4	0.59
ILMN_2843355	PCBP2	0.59
ILMN_2802979	SHH	0.59
ILMN_2519313	TMOD4	0.59
ILMN_2621921	CLCN7	0.59
ILMN_1250000	1500003O03RIK	0.60
ILMN_2769918	TIMP1	0.60
ILMN_2644350	THY1	0.60
ILMN_1225261	UCHL1	0.60
ILMN_3112873	TXNIP	0.60
ILMN_2918499	ABCB1B	0.60
ILMN_2862567	FNDC3B	0.60
ILMN_2732401	C130068O12RIK	0.60
ILMN_2857666	COL24A1	0.60
ILMN_2980663	AQP1	0.60
ILMN_1254523	GSTO1	0.60
ILMN_2718589	FCNA	0.60
ILMN_3083163	CP	0.60
ILMN_3125606	D12ERTD647E	0.60
ILMN_2687745	PNPLA2	0.61
ILMN_2869225	RPL19	0.61
ILMN_2438793	TMOD4	0.61
ILMN_2846904	MEST	0.61
ILMN_2658687	LTC4S	0.61
ILMN_2674575	MMD	0.61
ILMN_2672190	IDB1	0.61
ILMN_2655260	PTP4A3	0.61
ILMN_1227972	RPS16	0.61
ILMN_1221060	PPARG	0.61
ILMN_2705886	SCP2	0.61
ILMN_3121255	VEGFA	0.61
ILMN_2971559	EEF1A2	0.61
ILMN_2631161	FCRL3	0.61
ILMN_2885990	PDLIM3	0.61

ILMN_2463180	TNC	0.61
ILMN_2663832	FAU	0.62
ILMN_2705628	CLECSF8	0.62
ILMN_2877029	CYTL1	0.62
ILMN_1241214	MYH4	0.62
ILMN_3125966	KCNJ15	0.62
ILMN_2714638	MERTK	0.62
ILMN_2652500	LRG1	0.62
ILMN_1222246	ADFP	0.62
ILMN_1247811	ASS1	0.62
ILMN_2745555	MRPS21	0.62
ILMN_2771034	MFGE8	0.62
ILMN_3161554	VPS11	0.62
ILMN_2615250	MYOZ1	0.62
ILMN_1228657	FCGR2B	0.62
ILMN_3103896	TIMP1	0.62
ILMN_2742152	GADD45A	0.62
ILMN_2947568	GADD45A	0.63
ILMN_1223317	LGALS3	0.63
ILMN_2957161	SMYD2	0.63
ILMN_2637233	PRDX5	0.63
ILMN_2840985	ST6GALNAC5	0.63
ILMN_2694955	IGFBP4	0.63
ILMN_2614531	MCAM	0.63
ILMN_2613399	SCYE1	0.63
ILMN_2746895	GPR120	0.63
ILMN_2943165	AQP7	0.63
ILMN_2610771	NET1	0.63
ILMN_2630946	APRT	0.63
ILMN_2463181	TNC	0.64
ILMN_2770066	CYP4F18	0.64
ILMN_2968515	XPA	0.64
ILMN_1255047	C80638	0.64
ILMN_2721357	LHFPL2	0.64
ILMN_2626235	LGALS4	0.64
ILMN_2947292	MAP3K6	0.64
ILMN_2737302	CXCL12	0.64
ILMN_2624018	EEF1D	0.64
ILMN_2653215	NAGK	0.64
ILMN_1212636	STRAP	0.64
ILMN_1241390	4933408F15	0.64
ILMN_2874228	RPL12	0.64
ILMN_2872552	LOC622404	0.64

ILMN_1254987	MBNL2	0.64
ILMN_1231275	LOC381480	0.64
ILMN_2796798	5730469M10RIK	0.65
ILMN_1250213	SH3GLB1	0.65
ILMN_2596259	2410001C21RIK	0.65
ILMN_2971142	AMPD1	0.65
ILMN_2629663	E430002D04RIK	0.65
ILMN_2761109	CLIC4	0.65
ILMN_2687586	CXCL16	0.65
ILMN_1255556	PINK1	0.65
ILMN_2679937	PBXIP1	0.65
ILMN_2959863	NTN4	0.65
ILMN_2693403	ELA1	0.65
ILMN_1256257	G1P2	0.65
ILMN_1258526	LGALS3BP	0.65
ILMN_2740152	CHPT1	0.65
ILMN_1241695	MS4A6D	0.65
ILMN_2910934	CD52	0.65
ILMN_2668509	HP	0.65
ILMN_2451022	VIM	0.65
ILMN_2606295	GCH1	0.65
ILMN_1225727	CMA1	0.65
ILMN_2589785	CNN3	0.65
ILMN_2692615	TGM2	0.66
ILMN_2937735	IRAK2	0.66
ILMN_1235493	FXYD5	0.66
ILMN_3066763	ARL4	0.66
ILMN_2879506	2310037I24RIK	0.66
ILMN_3127391	NPR3	0.66
ILMN_3158250	CXCL12	0.66
ILMN_2753809	MMP3	0.66
ILMN_2712120	S100A6	0.66
ILMN_2754698	CD84	0.66
ILMN_1225658	UBL3	0.66
ILMN_3162925	LOC620078	0.66
ILMN_2705881	SCP2	0.66
ILMN_2653617	CD63	0.66
ILMN_2422333	TRAP1	0.66
ILMN_2952098	A930034L06RIK	0.66
ILMN_2721337	G6PDX	0.66

Table S2: Differentially expressed sciatic nerve genes between young and aged under naive conditions. Red: genes over-expressed in young greater than 1.5-Fold; Blue: genes over-expressed in aged greater than 1.5-Fold (less than 0.66).

IlluminaProbeID	GeneSymbol	Fold Change (young injured vs. aged injured)
ILMN_1253178	ALDH3A1	4.73
ILMN_2875730	MUP1	3.73
ILMN_2621448	ADH7	3.24
ILMN_1244618	DLK1	2.69
ILMN_3065459	MUP2	2.64
ILMN_1226472	RETNLA	2.44
ILMN_2638114	PTN	2.44
ILMN_1236788	IGFBP2	2.31
ILMN_2929896	PBK	2.10
ILMN_2607377	ITM2A	2.06
ILMN_1248788	0610005K03RIK	2.02
ILMN_3127335	SYTL2	2.02
ILMN_1216661	COL6A2	1.92
ILMN_2930897	IGFBP2	1.89
ILMN_2841593	FGFBP3	1.89
ILMN_3160486	2310037P21RIK	1.88
ILMN_2676379	PON1	1.84
ILMN_2681241	BIRC5	1.81
ILMN_3143404	MUP2	1.77
ILMN_2653207	TEAD2	1.75
ILMN_2998813	SEMA4F	1.73
ILMN_1247933	LYZ	1.71
ILMN_2513826	TNFRSF11B	1.70
ILMN_2734097	BTC	1.70
ILMN_2602139	CCL21A	1.69
ILMN_2836654	HIST1H2AO	1.67
ILMN_2970623	CENPP	1.67
ILMN_2612206	CDC20	1.67
ILMN_1218967	KIF2C	1.66
ILMN_2646296	D4BWG0951E	1.66
ILMN_2663328	2600001J17RIK	1.65
ILMN_1240125	GNB4	1.65
ILMN_2756421	LRRC15	1.65
ILMN_2977404	TMOD1	1.64
ILMN_1259610	6030411F23RIK	1.64
ILMN_2757125	PRC1	1.63
ILMN_2847144	HIST1H2AK	1.63
ILMN_1212907	D430039N05RIK	1.62
ILMN_2830661	TOP2A	1.62
ILMN_1255053	PLK1	1.61

ILMN_1234990	GAP43	1.61
ILMN_2599385	EPHA5	1.60
ILMN_2953807	LOC620807	1.60
ILMN_2867899	CDC20	1.59
ILMN_2603699	BIRC5	1.59
ILMN_2729289	MATN4	1.59
ILMN_2595597	ANLN	1.59
ILMN_1227352	ADAMTS19	1.59
ILMN_2745930	TAGLN3	1.58
ILMN_2593631	3000004C01RIK	1.58
ILMN_2753497	CCNB1	1.58
ILMN_2947568	GADD45A	1.58
ILMN_3123864	KCNH2	1.57
ILMN_2901180	SPC25	1.57
ILMN_2600744	RGS16	1.57
ILMN_2657844	CDC2A	1.57
ILMN_1245789	CCNE2	1.56
ILMN_1259388	COL6A1	1.56
ILMN_2669172	4930547N16RIK	1.56
ILMN_3132588	D930038M13RIK	1.55
ILMN_2643049	CHST8	1.55
ILMN_2785104	SFRS7	1.55
ILMN_1223045	2810417H13RIK	1.55
ILMN_1247358	EMID2	1.55
ILMN_3161959	E130016E03RIK	1.54
ILMN_2795040	HIST1H2AD	1.54
ILMN_2649291	LIP1	1.54
ILMN_1255960	SPC25	1.54
ILMN_2878501	NEDD4L	1.54
ILMN_2867901	CDC20	1.54
ILMN_1237399	RIT2	1.54
ILMN_2778872	MAD2L1	1.53
ILMN_2797642	NCAPH	1.52
ILMN_2604226	SEMA5A	1.52
ILMN_2656854	MYO6	1.52
ILMN_2642743	FGF5	1.52
ILMN_2762326	KIF22	1.52
ILMN_2900557	KIF15	1.52
ILMN_2588362	CDCA8	1.51
ILMN_2632712	BIRC5	1.51
ILMN_1220039	KIF4	1.50

ILMN_2875585	PLF2	0.23
ILMN_2996648	MRPPLF3	0.28
ILMN_2772632	SAA3	0.37
ILMN_2991799	CPA3	0.37
ILMN_2710905	S100A8	0.38
ILMN_1240746	MCPT4	0.40
ILMN_2760800	CXCL14	0.40
ILMN_2659426	CXCL14	0.42
ILMN_2863390	FCER1A	0.42
ILMN_1241350	VSIG4	0.43
ILMN_2997068	CMA1	0.43
ILMN_2642800	SOSTDC1	0.43
ILMN_2803674	S100A9	0.44
ILMN_2698449	DTR	0.44
ILMN_3158250	CXCL12	0.44
ILMN_2760019	CXCL13	0.45
ILMN_1242457	FPR-RS2	0.46
ILMN_2737302	CXCL12	0.46
ILMN_2794342	PMP2	0.46
ILMN_2817329	MPZ	0.46
ILMN_1223257	CCL4	0.46
ILMN_2965903	HDC	0.47
ILMN_3154269	PRX	0.47
ILMN_1246139	CLDN11	0.47
ILMN_2609590	CPA3	0.49
ILMN_2595664	DHH	0.49
ILMN_2712075	LCN2	0.50
ILMN_2656748	DFY	0.50
ILMN_2668510	HP	0.50
ILMN_2668509	HP	0.50
ILMN_1225727	CMA1	0.51
ILMN_2756046	GPR43	0.52
ILMN_1246800	SERPINA3N	0.52
ILMN_2593225	PRX	0.53
ILMN_2753809	MMP3	0.53
ILMN_3154691	SIRPB1	0.53
ILMN_2959291	UPP1	0.54
ILMN_2860804	TFRC	0.54
ILMN_2620406	NPTX2	0.54
ILMN_3161036	MCPT6	0.55
ILMN_3157658	CD209B	0.55
ILMN_2944824	HP	0.55
ILMN_2619848	TFRC	0.55

ILMN_2899041	MMP3	0.56
ILMN_2903169	GM484	0.56
ILMN_2995688	EG433016	0.57
ILMN_1214483	CRABP2	0.57
ILMN_2738082	ACDC	0.57
ILMN_2658908	CXCL12	0.57
ILMN_2661299	PMP22	0.57
ILMN_1249941	GDF10	0.58
ILMN_2622671	ACSL1	0.58
ILMN_2704576	MMP3	0.58
ILMN_1228535	PMP22	0.58
ILMN_1220697	NOTCH4	0.59
ILMN_2712986	CHI3L3	0.59
ILMN_2503052	TNNC1	0.59
ILMN_1224029	MAL	0.59
ILMN_2602352	MS4A6D	0.59
ILMN_1212619	4933428A15RIK	0.59
ILMN_2777498	IL1B	0.60
ILMN_1250421	MMP12	0.60
ILMN_1238886	CCL8	0.60
ILMN_2954987	MB	0.60
ILMN_2666018	MGLAP	0.60
ILMN_2692644	BC054059	0.61
ILMN_2443330	TTR	0.61
ILMN_2778655	VCAM1	0.61
ILMN_2595593	FXYD3	0.61
ILMN_1222679	CIDEC	0.61
ILMN_2661289	CSPRS	0.61
ILMN_2774160	HSD11B1	0.61
ILMN_2593774	1190002H23RIK	0.61
ILMN_2675268	4933408F15	0.62
ILMN_2731407	GDF3	0.62
ILMN_2614655	GPNMB	0.62
ILMN_2628629	CDH1	0.62
ILMN_3136638	SNCA	0.62
ILMN_2952275	ARG1	0.62
ILMN_1225376	SMOC1	0.63
ILMN_1253919	CCL3	0.63
ILMN_3078388	CD209B	0.63
ILMN_1259747	9230117N10RIK	0.63
ILMN_2690603	SPP1	0.63
ILMN_2614759	TIMP3	0.64
ILMN_2944601	4933439C20RIK	0.64

ILMN_2609182	6530401D17RIK	0.64
ILMN_2485323	TRF	0.64
ILMN_2738825	ACTA1	0.64
ILMN_3117876	CHI3L3	0.64
ILMN_2625893	CES3	0.65
ILMN_2727153	CLU	0.65
ILMN_2507286	TNFSF13B	0.65
ILMN_3049559	C4B	0.65
ILMN_2725927	SERPINA3G	0.65
ILMN_1241695	MS4A6D	0.65
ILMN_2654700	DRP2	0.65
ILMN_3115917	HSD11B1	0.65
ILMN_2616565	SLC2A3	0.65
ILMN_1226099	TNFSF13B	0.65
ILMN_3103896	TIMP1	0.65
ILMN_2741063	2810003C17RIK	0.66
ILMN_1232716	CYP1B1	0.66
ILMN_2593187	EGFL8	0.66

Table S3: Differentially expressed sciatic nerve genes between young and aged after injury. Red: genes over-expressed in young greater than 1.5-Fold; Blue: genes over-expressed in aged greater than 1.5-Fold (less than 0.66).

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